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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/87, A01K 67/027, C12N 15/12 — A2

(11) International Publication Number: WO 94/23049

(43) International Publication Date: 13 October 1994 (13.10.94)

(21) International Application Number: PCT/US94/03619

(22) International Filing Date: 1 April 1994 (01.04.94)

(30) Priority Data:
08/042,390 2 April 1993 (02.04.93) US

(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
Without international search report and to be republished upon receipt of that report.

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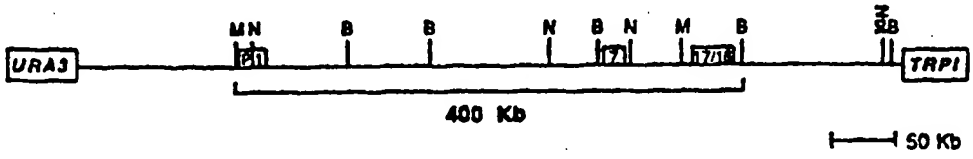
(54) Title: THE INTRODUCTION AND EXPRESSION OF LARGE GENOMIC SEQUENCES IN TRANSGENIC ANIMALS



(57) Abstract

This invention provides a method for the efficient introduction of cloned, very high molecular weight DNA into the germline of mice, whereby large genes can be expressed appropriately in transgenic mice. The β-amyloid precursor protein (APP) is known to be a complex gene consisting of 18 exons with total size estimates greater than 170 kb encoding three major RNA splicing forms. According to this invention, a neomycin resistance cassette is introduced into one of the arms of a 650 kb yeast artificial chromosome (YAC) which contains the entire unrearranged APP gene within 400 kb. Following gel purification, the YAC is introduced into embryonic stem (ES) cells by lipid mediated transfection. Neomycin resistant ES lines are isolated with the human APP gene stably integrated in an unrearranged state and expressing properly initiated and spliced full length human APP mRNA and APP human protein. Mouse chimeras generated from these ES lines transmit the YAC to their offspring, generating novel APP YAC transgenic mice. These transgenic mice express human APP gene products at significant levels in brain and peripheral tissues that mirror the expression of endogenous mouse APP gene products. This procedure will have great utility for transgenic studies of gene expression involving large genes and gene complexes.

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THE INTRODUCTION AND EXPRESSION OF LARGE GENOMIC SEQUENCES IN TRANSGENIC ANIMALS

The work leading to this invention was supported in part by Grant No. HD 24605 from the National Institutes of Health. The U.S. Government retains certain rights in this invention.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention concerns the introduction of large genomic sequences into the mammalian germline and their expression. The present invention also concerns transgenic mice having increased amyloid precursor protein gene dosage that mimics the trisomic condition that prevails in Down's Syndrome, generating an animal model of β-amyloidosis prevalent individuals with Alzheimer's Disease and Down's Syndrome.

Review of Related Art

A principal pathological hallmark of aged individuals and patients with Alzheimer's Disease (AD) or Down's Syndrome (DS) are deposits of the β-amyloid protein (Aβ) in the parenchyma of amygdala hippocampus, and neocortex. Aβ, a small peptide of between 39 and 42 amino acids, is derived from the amyloid precursor protein (APP), an integral membrane glycoprotein encoded by a large gene located on chromosome 21 (Goldgaber et al., 1987, *Science*, 235:877-880; Kang, et al., 1987, *Nature* (London), 325:476-478; Tanzi, et al., 1987, *Science*, 235:1120-1126). Alternative splicing of APP pre-mRNAs gives rise to at least four transcripts that encode Aβ-containing proteins of 695, 714, 751, and 770 amino acids (Kang, et al., 1987; Ponte, et al., 1988, *Nature*

(London), 331:525-532; Golde, et al., 1990, *Neuron*, 4:253-267). APP-751 and -770 contain a domain that shares homology with the Kunitz class of serine protease inhibitors (KPI domain) (Kitaguchi, et al., 1988, *Nature* (London), 331:530-532; Ponte, et al., 1988). Aβ is comprised of 11-15 amino acids of the transmembrane domain, and 28 amino acids of the extracellular domain of APP (Glenner and Wong, 1984, *Biochem. Biophys. Res. Commun.*, 120:885-890; Masters, et al., 1985a, *EMBO J.*, 4:2757-2763; Masters, et al., 1985b, *Proc. Natl. Acad Sci, USA*, 82:4245-4249). *In vitro*, APPs mature through a constitutive secretory pathway (Weidemann, et al., 1989, *Cell*, 57:115-126). APPs bound to the plasma membrane are substrates for endoproteolytic cleavage (Sisodia, 1992, *Proc. Natl. Acad. Sci., USA*, 89:6075-6079) within the Aβ region resulting in secretion of the ectodomain (Esch, et al., 1990, *Science*, 248:1122-1124; Sisodia, et al., 1990, *Science*. 248:492-495; Anderson, et al., 1991, *Neurosci. Lett.*, 128:126-128; Wang, et al., 1991, *J. Biol. Chem.*, 266:16960-16964); APP can also be reinternalized and processed by endosomal/lysosomal pathways (Cole, et al., 1989, *Neurochem Res.*, 14:933-939; Golde, et al., 1992, *Science*, 255:728-730; Haass, et al., 1992a, *Nature* (London), 357:500-503). Recent studies have documented the presence of Aβ-related peptides in the media of cultured cells and in human cerebrospinal fluid (Haass, et al., 1992b, *Nature* (London), 359:322-325; Seubert, et al., 1992, *Nature* (London), 359:325-327; Shoji, et al., 1992, *Science*, 258:126-129).

Postulated mechanisms involved in the generation of Aβ and events responsible for the formation of amyloid *in vivo* are not yet well defined (Selkoe,

1991, *Neuron*, 6:487-498; Price, et al., 1992, *Am. J. Pathol.*, 141:767-772; Sisodia and Price, 1992, *Curr. Opin. Neurobiol*, 2:648-652), but include: overexpression of APP (as occurs in trisomy 21), alterations in the efficiency of secretory versus endocytic maturation of APP, or changes in the stoichiometry of APP695 versus 751/770 expression in affected cells. The demonstration that a number of mutations in the APP gene are tightly linked to the presence of disease/amyloid deposition in some cases of familial AD (FAD) (Chartier-Harlin, et al., 1991, *Nature* (London), 353:844-846; Goate, et al., 1991, *Nature* (London), 349:704-706; Murrell, et al., 1991, *Science*, 254:97-99; Naruse, et al., 1991, *Lancet*, 337:978-979; Mullan, et al. 1992, *Nature Genetics*, 1:345-347) and in patients with hereditary cerebral hemorrhage with amyloidosis, Dutch type (HCHWA-D) (Levy, et al., 1990, *Science*, 248:1124-1126; Van Broeckhoven, et al., 1990, *Science*, 248:1120-1126; Hendriks, et al., 1992, *Nature Genetics*, 1:218-221) provides compelling evidence for the involvement of APP in the etiology of diseases in these individuals. Cells transfected with APP genes containing a double mutation present in a Swedish family with AD secrete high levels of Aβ-containing peptides into the culture media, suggesting that this mutation may alter the processing of APP *in vivo*.

Although transgenic strategies have been used to test some of the hypothetical mechanisms of amyloidogenesis, these efforts have failed to produce mice that recapitulate the principle features of AD (Sisodia and Price, 1992). Because *in situ* hybridization studies suggested that levels of APP-751 mRNA were elevated in hippocampal neurons of individuals with AD (Johnson, et al., 1990.

Science, 248:854-857); Quon, et al., 1991, *Nature* (London), 352:239-241) generated transgenic mice that expressed APP-751 under the transcriptional control of a neuron-specific enolase promoter. The absolute levels of transgene-deprived products was not fully documented and the transgenic animals exhibited poorly resolved extracellular Aβ deposits in hippocampus and cortex. Transgenic animals harboring a construct encoding the Aβ peptide under the transcriptional control of 2.8 kb (kilo base pairs) of the human APP promoter appeared to show small clusters of Aβ reactivity in the hippocampus of mice (Wirak, et al., 1991b, *Science*, 253:1-2), but subsequent studies showed that the patterns of immunoreactivity were nonspecific (Jucker, et al., 1992, *Science*, 255:1443-1445), and the paper was retracted. Two groups introduced transgenes encoding the carboxy-terminal 100 (CT-100) amino acids of human APP (Kawabata, et al., 1991, *Nature*, 354:476-478; Kammesheidt, et al., 1992, *Proc. Natl. Acad. Sci. USA*, 89:10857-10861). When the human Thy-1 promoter was used to drive transgene expression, CT-100 mRNA was detected (Kawabata, et al., 1991), but interpretation of the brain pathology was flawed and the paper was subsequently retracted. Mice with CT-100 drive by a brain dystrophin promoter had virtually undetectable transgene product and the cellular alterations did not reproduce the features of AD (Kammesheidt, et al., 1992).

The transgenic animals containing fragments of the APP genomic region do not reproduce the features of AD or DS, and thus, the need remains for a good model of AD and DS. The art has not been able to provide a transgenic animal containing the entire human genomic region associated with APP including control regions and introns.

To introduce large genomic regions into mice, investigators have previously used several technologies involving pronuclear microinjection of: microdissected chromosomes (Richa and Lo, 1989, *Science*, 245:175-177), overlapping genomic fragments of the human serum albumin gene that were subsequently recombined *in vivo* (Pieper, et al., 1992, *Nucl. Acid. Res.*, 20:1259-1264), a 70 kb β globin transgene generated by the *in vitro* ligation of two 35 kb cosmids, and a 35 kb yeast artificial chromosome (YAC) containing the tyrosinase gene (Schedl, et al., 1992, *Nucl. Acid. Res.*, 20:3073-3077). To transfer YACs into mammalian tissue culture cells, previous investigators have utilized a variety of techniques, including spheroplast fusion (Pachnis, et al., 1990, , *Proc. Natl. Acad. Sci. USA*, 87:5109-5113; Pavan, et al., 1990, *Mol. Cell. Biol.*, 10:4163-4169; Huxley, et al., 1991, *Genomics*, 9:742-750), lipofection (Gnirke, et al., 1991, *EMBO*, 10:1629-1634), calcium phosphate transfection (Eliceiri, et al., 1991, *Proc. Natl. Acad. Sci. USA*, 88:2179-2183), and electroporation (Fernandex-Luna, et al., 1991, *Genomics*, 10:756-764). Unfortunately, however, substantial portions of the yeast genome was also transferred (Pavan, et al., 1990, Gnirke, et al., 1991, and Huxley, et al., 1991). These methods may be adequate for introduction of YACs into somatic cells, but transfer of the yeast genome would be undesirable for propagation through the mammalian germline. Strauss and Jaenisch (1992), *EMBO J.*, 11:417-422, have gel-purified an 150 kb YAC containing the 24 kb *Mus. sprenus Collal* gene and introduced it into mouse fibroblasts by lipofection. However, they did not introduce their YAC into ES cells or into transgenic mice.

Thus, the need remains for a method of producing transgenic animals containing large, contiguous segments of the human genome. In particular, a need remains for a transgenic animal containing the entire human genomic region associated with APP, and capable of expressing that gene.

SUMMARY OF THE INVENTION

It is an object of this invention to provide a method for introduction of large, contiguous segments of foreign DNA into mammalian cells and into transgenic mammals.

It is another object of this invention to provide a transgenic mammal which expresses the human amyloid precursor protein.

This invention provides, in one embodiment, a method for producing a transgenic animal containing a contiguous foreign DNA segment of at least about 70,000 base pairs (70 kb), comprising:

(a) integrating, into a yeast artificial chromosome containing said contiguous foreign DNA segment, a selectable resistance cassette containing a first selectable marker which is expressed in yeast and a second selectable marker which is expressed in mammalian cells;

(b) separating the resultant yeast artificial chromosome containing both a contiguous foreign DNA segment and a selectable resistance cassette from other yeast genomic DNA;

(c) introducing the purified yeast artificial chromosome into embryonic stem cells;

(d) selecting a clone of embryonic stem cells containing the yeast artificial chromosome;

(e) injecting embryonic stem cells of the clone into blastocysts of the same species; and

(f) generating chimeric animals from the injected blastocysts.

In another embodiment, this invention provides a transgenic mouse containing at least about 400 kb of human genomic DNA, where the DNA comprises the human genomic region encoding the amyloid precursor protein (APP) promoter, with all 18 exons and the intervening sequences of the APP sequence.

Earlier studies had estimated the human APP gene to be 170 kb (Yoshikai, et al., 1990, *Gene*, 87:257-263) and it is unlikely that such a large DNA fragment will withstand the manipulations of standard pronuclear microinjection technologies. Therefore, a novel technology suitable for introduction of large DNA fragments into the mouse germline has been devised by the inventors. A yeast artificial chromosome (YAC) harboring the entire unrearranged 400 kb gene encoding human APP was introduced into embryonic stem (ES) cells and these cells were used to generate mice harboring exogenous human APP sequences in the mouse germline. In accordance with the present invention, the entire genomic region encoding human APP was introduced into the mouse germline. The APP genomic sequences contain transcriptional regulatory elements required for proper spatial and temporal expression, and appropriate splice donor and acceptor sites used to generate the entire spectrum of alternatively spliced APP transcripts.

This invention provides a model system which allows a direct test of the effects of APP dosage imbalance, mimicking the trisomic condition for APP occurring in individuals with DS. Using the method of this invention, the YAC

containing the human APP gene was stably integrated into mouse chromosomes, and the constitutive expression of alternatively spliced APP mRNA and encoded polypeptides was demonstrated in both ES cells and mice. Human APP is expressed in the transgenic mice at high levels relative to endogenous mouse APP both in brain and peripheral tissues.

These studies provide the first example of the introduction and expression of a DNA fragment larger the ~70 kb into the mouse germline (Strouboulis, et al., 1992, *Genes Dev.*, 6:1857-1864). The technique provided herein has broad applicability in mammalian genetics. For example, transgenic animals prepared according to the method of this invention may be used for the examination of the developmental expression of contiguous genes, i.e., homeobox sequences and globin sequences, or the examination of cis-acting elements that act at great distances from the native promoter. Permutations of this approach will be invaluable in producing animal models of genetic disorders, including AD and DS.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows pulsed-field gel analysis of APP YACs. Yeast chromosome-size DNA molecules from yeast strain AB1380 (lane 1) and from nine different AB1380 yeast transformants that contain YACs with APP sequences (lanes 2-10) were separated across a 1% agarose gel by pulsed-field gel electrophoresis (PFGE). Shown on the right are approximate molecular weights in kilbases of the various APP YACs displayed in Table I.

Figure 2 A-C shows representative restriction analysis of the APP YACs, where yeast chromosome-size DNA from 8 of the 9 yeast transformants that contain YACs with APP sequences, digested with either HindIII (lanes 1-8) or

EcoRI (lanes 11-18), were fractionated on 1% agarose gels for Southern analysis. Controls include a yeast transformant with a SODI containing YAC (SOD-8, lanes 9 and 19), and human DNA (lanes 10 and 20). The Southern blots shown are probed with: (A) DNA fragment containing an upstream portion of the human promoter (343 bp EcoRI/HphI fragment of pMTI-2307; Wirak, et al., 1991a, *EMBO, J.*, 10:289-296; (B) DNA fragment containing human APP exon 7 (~180 bp EcoRI/HindIII fragment of pID; (C) DNA fragment containing 1/2 of exon 16, exon 17, and exon 18 from a mouse APP cDNA (1 kb EcoRI fragment). The presence of multiple bands in A corresponds to a small contamination of the promoter probe with a human *Alu* element. ΦX174 HaeIII-digested DNA markers are shown on the right.

Figure 3 A and B shows genomic maps of the 650 kb YAC, APP-8. (A) A genomic map of APP-8 generated by partial digestion of yeast chromosome-size DNA probing Southern blots with sequences specific for the *URA3* or *TRP1* arm of the YAC and for APP sequences including the promoter (P, exon 7, and exons 17 and 18; (B) A representative Southern blot of partial digests of APP-8. Undigested APP-8 (lanes 1, 7, 13, and 19), or APP-8 digested with increasing amounts of Not I (lanes 2-6), NruI (lanes 8-12), MluI (lanes 14-19), and BssHII (lanes 20-24) fractionated by PFGE are shown. DNA was transferred to nylon membranes and then hybridized with sequences specific for the *TRP1* arm of the YAC (2.3 kb EcoRI/PvuII fragment of pBR322). The solid arrow represents the size of the undigested 650 kb YAC, and the dotted arrow represents a band often observed in APP-8 that probably represents a deletion product of the YAC during propagation in yeast. Shown on the left are the molecular weights in kilobases.

Figure 4 A-D shows integration of a neomycin resistance cassette into the 650 kb YAC, APP-8. Figure 4A is a diagrammatic representation of the integration of the pHIS3PyF101neobpA vector into the *TRP1* arm of APP-8. pHIS3PyF10neobpA was digested with ScaI (S) site and introduced into yeast strain YPH857 containing APP-8. The vector homologously recombines with the *amp*^{r} gene in the *TRP1* arm of the YAC, and His+ transformants are selected. DNA arrangements of the original YAC arm, and a single or multiple copy integration of the vector are shown from top to bottom. When digested with NotI (N), these different DNA arrangements yield the fragment sizes predicted on the right, with increasing amounts of the 5.5 kb fragment indicative of a concomitant increase in copy number of the integrating vector. Figure 4B shows a Southern blot of four His+ transformants (lanes 1-4), the original APP-8 YAC in yeast strains YPH857 (lane 5) and AB1380 (lane 6), and a control yeast strain YPH857 (lane 7), digested with NotI, fractionated on 1% agarose gels, and probed with the *amp*^{r} gene (2.3 kb EcoRI/PvuII fragment of pBR322). On the right are shown the sizes of molecular weight markers (kb) of λ DNA and λ HindIII-digested DNA, and on the left sizes of the fragments predicted in A. In Figure 4C, the Southern blot shown in B was reprobed with the *neo*^{r} gene (834 bp EcoRI/XbaI fragment of pPo12sneobpA). Figure 4D is a diagrammatic representation of the pHIS3PyF101neobpA vector.

Figure 5 A-D shows PCR analysis of G418^r ES cell lines transfected with the 650 kb YAC, Py.8. A no DNA control (lane 2), DNA from untransfected ES D3 cells (Lane 3), Py.8 DNA in yeast (lane 4), human DNA (lane 5), and DNA from 5 G418^r ES lines (lanes 6-10) were subjected to PCR with two sets of

primers per reaction. One primer set amplified a mouse-specific 203 bp product (Xist) as a positive control (dotted arrow), while the others (solid arrow) amplified product specific for the Py.8 YAC including: a 277 bp *URA3* product (A); a 400 bp human APP promoter product (B); a 411 bp human-specific APP exon 7 product (C); and a 319 bp human-specific APP exon 17 product (D). PCR products were fractionated on 2% agarose gels and stained with ethidium bromide. The promoter-specific PCR products shown in B were confirmed by Southern analysis. The ΦX174 HaeIII-digested molecular weight markers are shown in lane 1.

Figure 6 shows *Alu* element profiles of APP YAC transfected ES cell lines. High molecular weight DNA from Py.8 in yeast strain YPH857 (lane 1), YPH857 (lane 2), ES D3 cells (lane 3), ES D3 cells transfected with pHIS3PyF101neobpA (Py8.33, lane 4), and three ES lines positive for YAC DNA (lanes 5-7; Py8.2, Py8.9, and Py8.29) was digested with HindIII, before fractionation on a 0.8% agarose gel. After transfer, the Southern blot was hybridized to a human repetitive *Alu* element (284 bp HphI *Alu* fragment from pMTI2307). Arrowheads mark *Alu* elements deleted in Py8.29.

Figure 7 A and B shows fluorescent in-situ hybridization of APP YAC transfected ES cell lines. Metaphase chromosome spreads of ES lines Py8.2 (A) and Py8.9 (B) were hybridized to human COT-I DNA.

Figure 8 shows expression of human APP mRNA in YAC transfected ES cell lines. Cytoplasmic RNA, isolated from ES D3 cells (lane 1), the three ES lines positive for YAC DNA (lanes 2-4; Py8.2, Py8.9, and Py8.29), and ES D3 cells transfected with pHIS3PyF101neobpA (lane 5; Py8.33) was fractionated by

denaturing gel electrophoresis and transferred to nitrocellulose. The Northern blot was hybridized with a full length human APP-695 cDNA (A), stripped, and then rehybridized with mouse β-tubulin cDNA (B). The large arrowhead (A) represents full length APP mRNA, and the two smaller arrowheads (B) represents β-tubulin mRNAs that utilize alternative polyadenylation sites. Shown on the right (A) are the positions of 18S and 28S ribosomal RNA.

Figure 9 shows expression of human APP in YAC transfected ES cell lines. Protein extracts from a CHO line transfected with human APP-695 (lane 1) or -770 (lane 2) cDNA, ES D3 cells (lane 3), and the three ES lines positive for YAC DNA (lanes 4-6; Py8.2, Py8.9, and Py8.29) were fractionated by SDS-PAGE and then electrophortically transferred to nitrocellulose. The blot was hybridized with the human-specific APP antibody B5 (A) or a β-tubulin antibody (B). The arrowhead represents human APP expressed in ES cell line Py8.2.

Figure 10 A-D shows PCR analysis of agouti offspring from a Py8.9 chimera. A no DNA control (lane 2), DNA from untransfected ES D3 cells (lane 3), Py.8 YAC DNA in YPH8587 (lane 4), human DNA (lane 5), ES cell line PY8.9 DNA (lane 6), DNA from a C57BL6/J mouse (C9.3, lane 7), DNA from a Py8.9 chimera (C9.6, lane 8), and from 8 agouti offspring of C9.6 (C9.13-C9.20, lanes 9-16), were subjected to PCR with two sets of primers per reaction. One set amplified a mouse specific 203 bp product (Xist) as a positive control (dotted arrow), while the others (solid arrow) amplified products specific for the Py.8 YAC including: a 277 bp URA3 product (C); and a 319 bp human-specific APP exon 17 product (D). PCR products were fractionated on 2% agarose gels

and stained with ethidium bromide. Molecular weight markers are shown in lane 1 and represent a HaeIII digest of ΦX174 DNA.

Figure 11 A-C shows RT-PCR analysis of APP mRNA expression in APP YAC transgenic mice. RNA was isolated from brain (B), heart (H), kidney (K), and testes (T) of a control mouse (C9.20) or an APP YAC transgenic mouse (C9.24) and was then subjected to RT-PCR analysis with primers corresponding to highly conserved sequences of human and mouse APP cDNA. PCR products were digested with SphI and fractionated on 2% agarose gels. Figure 11A shows an ethidium bromide-stained gel of RT-PCR products after SphI digestion. Markers are a 1-kb ladder (Bethesda Research Laboratories). Sizes of PCR products are shown on the right. Lanes 9-10 represent PCR products generated in the presence of human or mouse APP-695 cDNA templates, respectively. Figure 11B shows autoradiogram of the gel shown in Figure 11A. Labeled bands were excised and radioactivity determined by liquid scintillation counting. The ratio of human to mouse PCR products derived from tissue RNA in the transgenic animal is shown at the bottom of the panel. Figure 11C shows RT-PCR analysis of independent transgenic mice on an ethidium bromide-stained gel of SphI-digested RT-PCR products from brain RNA of three APP YAC transgenic mice (laces 2-4) derived from Py8.9 chimera C9.6 and a control animal (lane 1). Essentially identical levels of human (568 bp) and mouse (516 bp) specific products are observed in independent transgenic mice.

Figure 12 A and B shows RT-PCR analysis of alternative splicing of human and mouse APP transcripts in APP YAC transgenic mice. RNA isolated from brain (B), heart (H), kidney (K), and testes (T) of a control mouse (C9.20)

or an APP YAC transgenic (C9.24) was subjected to RT-PCR analysis with primers that flank the KPI-encoding exons. Figure 12A shows a schematic diagram of the RT-PCR analysis. Figure 12B shows an autoradiograph of the PCR products, digested with SphI to cleave mouse products specifically, fractionated on 2% agarose gels, dried, and exposed to x-ray film. Arrows in lane 1 represent mouse-specific products of 360, 528, and 585 bp representing mouse APP-695, -751, and -770 transcripts, respectively. Asterisks in lane 5 represent human specific products of 490, 658, and 715 by representing human APP-695, -751, and -770 transcripts, respectively.

Figure 13 A and B show the steady-state expression of human APP in APP YAC transgenic mice. Protein extracts were prepared from brain (B), heart (H), kidney (K), and testes (T), fractionated by SDS-PAGE, and subjected to Western blot analysis. Figure 13A is a Western blot of tissue extracts from control (C9.20) and transgenic (C9.24) tissues with antibody B5, specific for human APP. Figure 13B is a Western blot of tissue extracts with CT-15, an antibody raised against the common carboxy terminal 15 amino acids of human/mouse APP.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides a method whereby high molecular weight YAC DNA can be transfected into ES cells, stably integrated into specific mouse chromosomes, and transferred into the mouse germline in a highly efficient and reproducible manner. Large complex genes carried on such YACs can be expressed at levels and in tissues similar to endogenous gene products. Thus, the method of this invention may be used to analyze large gene complexes (i.e., homeobox or globin genes), higher order genomic structure (i.e., chromosome

inactivation, imprinting, and dosage imbalance), and gene expression (i.e., far acting cis sequences). Furthermore, this invention provides for the development of models of human disease, the identification of disease loci, and the design of new methods of gene therapy by providing transgenic animal models with large segments of human DNA associated with the respective diseases.

The disclosure herein assumes familiarity with the standard techniques for manipulation and analysis of recombinant DNA, as taught in, e.g., Sambrook, et al., "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory Press, 1989, or Ausubel, et al., "Current Protocols in Molecular Biology", Greene Publishing Assoc. and Wiley-Interscience, 1991. General yeast protocols, yeast media and standard genetic techniques are taught in Guthrie, et al., 1991, *Methods In Enzymology*, 194, Academic Press.

The method of this invention provides for incorporation of large, contiguous foreign DNA segments into mammalian cells and into transgenic animals. Prior to the present invention, technical limitations, including vector capacity and DNA handling techniques, had prevented the production of recombinant cells or transgenic animals containing contiguous segments of foreign DNA greater than about 70 kb. The method taught herein provides for production of recombinant mammalian cells, as well as transgenic animals, containing contiguous foreign DNA segments of greater than about 70 kb, preferably greater than about 150 kb and most preferably greater than about 400 kb. The contiguous DNA sequences contemplated herein are usually genomic DNA sequences, and include genes where the genomic sequence including control sequences is at least about 100 kb, or gene complexes or gene clusters, which may be incorporated in

a coordinated fashion. The foreign DNA can be obtained from any source of DNA that provides contiguous DNA segments of the size contemplated by this invention. The foreign DNA segments are cloned into a yeast artificial chromosome vector, and the size of the foreign DNA is limited only by the capacity of the yeast artificial chromosome system and the ability of the target mammalian cell to integrate additional DNA.

A preferred source for large contiguous DNA segments is a yeast artificial chromosome (YAC) library. Preparation of such libraries is well known in the art (Guthrie, et al.). Ready-made libraries and selected clones are available from the Chromosome 21 Joint YAC Screening Effort courtesy of Dr. David Patterson, Eleanor Roosevelt Institute for Cancer Research, 1899 Gaylord Street, Denver, CO 80206., Tel. No. (303) 333-4515 and Fax No. (303) 333-8423. YAC libraries may be screened by any of the techniques used in the art for screening DNA libraries, including probing with nucleic acid fragments of known sequence, primer directed amplification, such as PCR (polymerase chain reaction), and the like. The preferred method for selecting a YAC containing the desired large DNA segment is PCR screening. Separation of the YAC from the rest of the yeast genome by, e.g., pulsed-field gel electrophoresis (PFGE) permits analysis and characterization of the DNA insert. The skilled worker will readily appreciate that the DNA sequence of the isolated YAC can be analyzed using various techniques; particular techniques will be selected depending on the sequence and the information need to distinguish the DNA. Isolation, replication, cloning and analysis of YACs is within the skill of the art as taught in, e.g., Sambrook, et al., Guthrie, et al., and Ausubel, et al.

In a preferred embodiment of this invention, multiple selectable markers are incorporated into the YAC used for introducing the large foreign DNA segment into a transgenic animal. Selectable markers are DNA sequences which, when expressed, produce a biological effect that is readily detectable in the expressing organism. When DNA containing a selectable marker is introduced into a host cell, the host containing the marker is easily selected from similar cells which do not contain the marker. Selectable markers are well known in the art of molecular cloning, and choice of a particular marker is within the skill of the art.

Preferably, the selectable markers are integrated into the YAC by introduction of a cassette containing one or more selectable markers. More preferably, the selectable marker cassette used in the method of this invention contains at least one marker that is expressed in yeast, at least one marker that is expressed in mammalian cells and a restriction enzyme cleavage site flanked by sequences homologous to sequences in the vector used to create the YAC library. Either or both of the selectable markers may be present on the cassette in multiple copies.

Integration of the cassette is accomplished by linearizing the cassette with the restriction enzyme and introducing the linearized cassette DNA into yeast containing the YAC, where the cassette is integrated into the YAC by recombination at the homologous sequences (the integration site target). A particularly preferred cassette may be constructed as disclosed herein in Example 2 below. The preferred cassette contains a neomycin resistance marker under control of a mutant polyoma-enhancer/thymidine kinase of promoter and is especially effective in embryonic stem cells. Preferably the selectable marker

cassette is integrated into the YAC at an integration site target only in the vector, but that is not present in the foreign DNA sequence, to avoid disruption of the contiguous sequence of the foreign DNA. Successful integration can be confirmed by standard techniques, including, e.g., PFGE and restriction fragment analysis. The integrity of the foreign DNA segment following integration of the selectable marker cassette should also be confirmed usually by analyses similar to those used to characterize the YAC.

It is clear that YAC DNA can be easily manipulated in yeast prior to introduction into the mammalian germline. With this technique, it is possible to assess the contributions of insertions, deletions, or point mutations in genes associated with the etiology of specific diseases including AD, ALS, and Huntington's disease. These mutations can be introduced by controlled manipulation of the YAC DNA in yeast, and the resulting DNA can produce transgenic animals with known genetic modifications.

Preferably the YAC is purified from the yeast and the remaining yeast genome before it is introduced into the mammalian cell to limit the amount of foreign DNA (of potential detrimental effect) introduced into the mammalian cell. This is preferably accomplished by PFGE, to avoid mechanical damage to the YAC, which could disrupt the foreign DNA segment. Recovery of the YAC from the agarose gel following PFGE is enhanced by inclusion of an optimal concentration of polyamines to protect high molecular weight DNA from shearing. Investigation suggests that the larger the YAC, the higher the concentration of polyamines that is needed to protect the DNA. For example, 50-100 μM polyamines were required to keep an 180 kb SODI YAC intact, a finding similar

to that observed by Strauss and Jaenisch (1992), whereas the 650 kb APP YAC used in the present study required ~10 times higher concentration to protect the DNA from shearing.

It is desirable to provide a large amount of YAC DNA to the mammalian cell. The major limiting factor in this transfection protocol is the quantity of YAC DNA that can be isolated from preparative gels. Particularly preferred YAC amplification vectors are those with conditional centromeres; they allow for a 20-fold enrichment of the YAC relative to the yeast chromosomes (Smith, et al., 1990, *Proc. Natl. Acad. Sci. USA*, 87:8242-8246).

The YAC containing the large, contiguous foreign DNA segment, preferably containing multiple copies of selectable markers expressed in mammalian cells, is then introduced into a mammalian cell. The YAC may be introduced into a mammalian cell by any process known in the art, so long as the large, contiguous foreign DNA segment is not disrupted. A preferred method of introduction (or transfection) is lipid-mediated transfection or lipofection (see, e.g., Ausubel, et al.). Transfected mammalian cell lines can be selected which contain the selectable markers on the YAC using standard methods. Transfected cell lines that are positive for the selectable markers are then analyzed for the presence of the intact segment of the foreign DNA. Techniques for analysis of the DNA content of the transfected mammalian cells are similar to the techniques used for analysis of the integrity of the foreign DNA in the YAC and well known to the skilled worker, and they include PCR analysis of DNA extracts of the cells and fluorescent in situ hybridization, as well as Northern blot and Western blot

analysis to determine if the transfected cell expresses the protein encoded by the foreign DNA.

In the experiments described below, approximately ten G418^r colonies were obtained per 10^7 ES cells transfected with YAC DNA, with 10% of the resulting G418^r colonies containing a majority of the YAC. The fraction of successfully transfected cells containing the YAC was similar to the observed by Strauss and Jaenisch (1992) for fibroblasts. However, the efficiency of transfection achieved by the present method (for a YAC over four times larger than previously described) was almost five times higher than achieved by Strauss and Jaenisch. These results may, in part, be a reflection of the multiple *neo^r* cassettes that were integrated in the Py.8 YAC. Preliminary transfections with Py. 2 YAC DNA containing a single *neo^r* cassette (Figure 4) yielded 5-10 fold fewer G418^r colonies. Thus, an advantage of a targeting strategy in which selectable markers are introduced into the YAC vector arms is the frequent integration of multiple selectable markers, such as those contained in the *neo^r* cassettes, without interrupting the YAC insert DNA. The fraction of ES cells containing the YAC can be improved by introducing a second mammalian selectable gene, such as resistance to hygromycin, into the other YAC arm prior to introduction into cells.

Preferred mammalian cells for transfection are embryonic stem cells. Embryonic stem (ES) cells are pluripotential cells that are capable of colonizing a host embryo and contributing to the formation of all tissues, including the germ-line (Robertson, E.J., 1987, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, pp. 71-112, Oxford:IRL Press). ES cells have a normal chromosomal constituent, are rapidly dividing, and easily cultured and passaged.

Importantly, ES cells are also amenable to manipulations in culture, including gene transfer and drug resistance selection. Preferred embryonic stem cells are those of mouse (Bradley, A., 1987, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, pp. 113-151, E.J. Robertson ed., Oxford:IRL Press) or pig. Embryonic stem cells transfected as described herein are particularly useful for the construction of transgenic animals. In particular, transfected ES that express the foreign protein, as determined by analysis as described above, are preferred for production of transgenic animals according to the method of this invention.

Transgenic animals are prepared by injecting the transfected ES cell line obtained above into blastocysts of the same animal species as the ES cells, but from a strain having one or more easily detectable trait which is different from the strain from which the ES cells were derived. The injected blastocyst is allowed to develop into a chimeric animal, containing cells derived from both the blastocyst and the transfected ES cell line. Preferably this is accomplished by transferring the embryo (the injected blastocyst) into a pregnant or pseudopregnant female, where the embryos develop until the chimeric animals are born. Chimeric animals are selected by standard techniques. In a preferred embodiment, the embryonic stem cells and the blastocyst come from different strains of mouse, which differ in a readily detectable characteristic, such as coat color. Chimeric mice will exhibit at least some of the coat color characteristic of the ES source strain. Chimeric animals are mated to the source strain of the blastocyst and chimeric offspring are tested to determine whether they are carrying the foreign DNA. The offspring carrying the foreign DNA represent successful transmission through the germline. The method of this invention provides for the introduction of

extremely large, well-defined genomic sequences into mice with high efficiency, by the transfer of purified YACs, via lipid mediated transfection, into ES cells, and eventually into the germline of mice. This approach has several major advantages: the ability to completely characterize the genomic DNA carried in the YAC, the avoidance of extensive manipulation of large DNA molecules involved in microinjection, and the ability to easily identify and analyze ES cell that contain integrated YAC DNA prior to transfer into animals.

In a particular embodiment, this invention provides animals that test the hypothesis that overexpression of APP may lead to the deposition of Aβ, a pathological hallmark of individuals with AD and DS. Transgenic mice that overexpress the entire APP gene closely mimic the APP dosage imbalance observed in individuals with DS. Previous efforts have not been successful in reproducing the deposition of Aβ characteristic of AD and DS (Sisodia and Price, 1992). A consistent feature of these earlier efforts is that transgene products have been expressed at low levels compared to endogenous APP. Consequently, to date, these studies have provided little insight into the underlying molecular mechanisms of amyloidogenesis.

In contrast, large genomic sequences of interest can be introduced and expressed in mice by the method of this invention. Because transgenes have been limited to ~70 kb in transgenic animals, the introduction of a 650 kb YAC containing the entire 400 kb human APP gene into mice represents a very significant advance. Indeed, the 650 kb of introduced human DNA represents approximately 1.5% of all human chromosome 21q. Because this genomic fragment contains the entire APP gene, ~250 kb of flanking sequences, and

possibly additional unidentified genes, these animals have been termed APP YAC transgenic mice. In the APP YAC transgenic mice of this invention, a genomic region that contains the entire human APP gene and flanking sequences is introduced, and human APP mRNA and protein are expressed at significant levels in the brain as well as other tissues of these animals.

Finally, transgenic mice produced according to the method of this invention allow stringent testing of the roles of mutations in cases of early-onset FAD and HCHWA-D in amyloidogenesis. By homologous recombination in yeast, APP mutations are introduced into the 650 kb APP YAC so that the encoded polypeptides will harbor amino acid substitutions either in the APP transmembrane domain (position 717 of APP-770) or immediately upstream of the Aβ sequence (position 670 and 671 of human APP-770) found in FAD, or substitutions in the Aβ sequence (position 692 or 693 of APP-770) found in HCHWA-D. The double mutation (position 670 and 671) documented in a Swedish FAD kindred, increases the production of Aβ *in vitro* (Citron, et al., 19, *Nature* (London), 360:672-674; Xiao-Dan, et al., 1993, *Science*, 259:514-516). Tissue culture cells transfected with cDNAs encoding this mutant polypeptide secrete higher levels of Aβ-containing peptides than cells transfected with wild-type constructs. The introduction of a human YAC expressing mutant APP into ES cells and, subsequently, into the germline of transgenic mice according to this invention thus provides a direct test of the significance of mutations in the APP gene in the etiology/pathogenesis of AD and HCHWA-D.

EXAMPLES

The following Examples are provided to more fully illustrate the present invention. They are not intended to limit the invention described herein, which is only limited by the appended claims.

Example 1.

Characterization of APP YACs

A human YAC library was screened by PCR using primers specific for human APP exon 14 through the chromosome 21 Joint YAC Screening Effort and lead to the identification on nine independent YAC isolates. These YACs, designated as APP-1 through APP-9, were fractionated by pulsed-field gel electrophoresis (PFGE; Figure 1). The YACs varied in size from 150 kb (APP-3, lane 4) to greater than 1,000 kb (APP-2, lane 2).

General yeast protocols, yeast media, and standard genetic techniques are described in Guthrie and Fink, 1991.

Nine APP YACs were obtained from the Chromosome 21 Joint YAC Screening Effort by screening with PCR primers specific for human APP exon 14 (exon 14 forward, 5'-TTGAATGCCATGTGCCTCAG-3', and exon 14 reverse, 5'-GCTGAATTCCCCATTCACGG-3'). Clone numbers of each isolate are: A122A9 (APP-1); D110G1 (APP-2); B53B10 (APP-3); A223G10 (APP-4); D110G6 (APP-5); B171E3 (APP-6); D110E9 (APP-7); B142F9 (APP-8); and A167D10 (APP-9). The SOD-8 YAC is a 180 kb YAC containing the superoxide dismutase I gene that was transformed with a neomycin resistance cassette described previously (Pavan, et al., 1990).

The isolation of high molecular weight DNA from yeast was a modified procedure of that described previously (Anand, et al., 1989, *Nucl. Acids Res.*, 17:3425-3433) by scaling up and resuspending 50-100 ml of yeast grown to saturation in YPD, in a final volume of 6-8 ml, to obtain more concentrated plugs. Isolation of total genomic DNA from yeast followed a protocol described in Davis, et al. (1980), *Methods In Enzymology*, 65:404-411.

PFGE was performed on a Bio-Rad contour-clamped homogeneous electric field (CHEF) DR II apparatus, in 1% agarose (SeaKem LE, from FMC) for analytical gels, or 1% low melting point agarose (SeaPlaque, from FMC) for preparative gels, in 0.5 X TBE electrophoresed at 200 V (140-170 mA) at 12°C. Switching times varied from 60-90 seconds for various applications. A standard yeast karyotype was obtained with a 60 second pulse for 16 hours, followed by a 90 second pulse for 14 hours. Partial digests of the 650 kb APP YAC was performed by digesting yeast plugs with 0.001, 0.01, 0.05, 0.1, and 1 unit of BssHII, MluI, NruI, and NotI for 1 hour and terminating each reaction with 50 mM EDTA. Digests were fractionated by PFGE with a 60 second pulse for eight hours, followed by a 90 second pulse for 15 hours. Preparative gels for isolation of the 650 kb YAC were performed with a 60 second pulse for 44 hours.

Extensive restriction analysis of each APP YAC documented the genomic arrangement of inserted human sequences. DNA digests were analyzed by Southern analysis using several nonoverlapping probes which encompassed all 18 exons of the APP cDNA and the APP promoter. Figure 2 is a representative blot which demonstrates hybridization of YAC DNAs with probes representing different portions of APP: the human APP promoter (A), exon 7 (B), and a fragment

covering 1/2 of exon 16, exon 17, and exon 18 (C). The PFGE and restriction analysis (Table 1) shows that only the four largest YACs, containing inserts of 650 kb (APP-8), 850 kb (APP-5 and APP-7), and >1,000 kb (APP-2), contained all 18 APP exons as well as the APP promoter. Comparison of hybridization patterns to that obtained with human DNA, revealed that these YACs contained an unrearranged APP gene. Furthermore, the restriction map obtained for the APP YACs is entirely consistent with the map generated by analysis of cosmids containing human APP sequences (Yoshikai, et al., 1991, *Gene*, 102:291-292).

The analysis of the 650 kb APP-8 YAC was extended by partial digestion with rare cutting restriction enzymes, fractionation by PFGE, and Southern analysis with YAC and human APP-specific probes. Figure 3B documents the analysis of partial digests with NotI (lanes 2-6), NruI (lanes 8-12), MluI (lanes 14-18), and BssHII (lanes 20-24) probed with sequences specific for the TRPI arm of the YAC. A tentative genomic map of the insert in APP-8 is shown in Figure 3A. Notably, our PFGE map indicates that the entire APP gene is approximately 400 kb, substantially larger than the minimal size estimate of 170 kb generated by cosmid analysis (Yoshikai, et al., 1990).

Table 1.

APP YAC CLONES	SIZE OF YAC	APP SEQUENCES		
		PROMOTER	EXONS 1-8	EXONS 9-18
APP-1	200 Kb	-	-	+
APP-2	1,000 Kb	+	+	+
APP-3	150 Kb	-	-	+
APP-4	200 Kb	-	-	+
APP-5	850 Kb	+	+	+
APP-6	210 Kb	-	-	+
APP-7	850 Kb	+	+	+
APP-8	650 Kb	+	+	+
APP-9	340 Kb	-	-	+

The presence (+) or absence (-) of unrearranged APP sequences in the YACs was determined by extensive restriction analysis. Southern blots of these digests (BamHI, EcoRI, HindIII, and all combinations of these enzymes) were hybridized with 2-3 exon pieces of the mouse APP-695 cDNA, a fragment containing human exon 7, and a fragment containing the human APP promoter. Representative Southerns are shown in Figure 2.

Example 2.

Manipulation of APP YACs

APP-8 was selected for our initial studies because this YAC appeared to contain the entire unrearranged human APP gene. As a first step towards introducing this YAC into ES cells, APP-8 was transferred by spehorplast transformation from yeast strain AB1380 to YPH857, a versatile yeast strain that permits extensive manipulation of YACs. The next step in our strategy involved the insertion of neo^r cassettes into the TRPI arm of APP-8, by taking advantage of the presence of the his3-Δ200 marker in YPH857. This strategy differs substantially from previous efforts which relied on integration of neo^r cassettes into YACs through a human repetitive *Alu* element (Pavan, et al., 1990). Concerned with the presence of several *Alu* elements within the APP gene itself (Salbaum, et al., 1988, *EMBO J.*, 7:2807-2813), we predicted that the earlier targeting strategies could lead to APP gene disruption.

To introduce a neo^r cassette into the TRPI arm of any YAC (cloned in pYAC4), we constructed the integrating plasmid pHIS3PyF101neobpA, that consists of a neo^r cassette for positive selection in ES cells, and the yeast HIS3 gene for positive selection in yeast. The YAC targeting vector phis3PyF101neobpA was constructed as follows. An XhoI 1347 bp fragment containing pol2sneobpA (Soriano, et al., 1991, *Cell*, 64:693-702) was first subcloned into pBluescript II KS-. The pol2 promoter was removed by digestion with EcoRI and religation, creating the promoterless pneobpA. pneobpA was digested with EcoRI, the ends filled in with Klenow DNA polymerase and dNTPs. In parallel the mutant polyoma enhancer PyF101 (Linney and Donerly, 1983, *Cell*.

35:693-699) driving the herpes simplex virus thymidine kinase promoter (TK) was isolated on a 340 bp XbaI/BglII fragment from pPyF101TKCAT (Lamb, et al., 1991. *Gene Expression*, 1:185-196) and the ends filled with Klenow DNA polymerase. The pneobpA vector was ligated to the polyoma-enhancer/TK-promoter fragment, creating pPyF101neobpA. Finally, the HIS3 gene of yeast was isolated on a 1157 bp BamHI fragment from p288, blunt ended and cloned into the SmaI site of pPyF101neobpA.

The 650 kb APP YAC was transferred from yeast strain AB1380 (MATa, φ+, ura3-52, trp1, ade2-1, can1-100, lys2-1, his5) to YPH857 (MATα, φ-, ura3-52, trp1-Δ63, lys2-801, ade2-101, his3-Δ200, leu2-Δ1, cyh2^r; by spheroplast transformation as described previously (Connelly, et al., 1991, *Genomics*, 10:10-16). The phis3PyF101neobpA plasmid was targeted to the TRPI arm of the YAC by first digesting the plasmid with ScaI, which linearizes the plasmid within the amp^r gene. Linearized plasmid (3 μg) was then transferred to YPH857 containing the 650 kb APP YAC by lithium acetate transformation (Ito, et al., 1983, *J. Bacteriol.*, 153:163-168). His$^+$ transformants were isolated and screened by PFGE and restriction analysis.

The 650 kb APP YAC with multiple copies of phis3PyF101neobpA insertions in the arm (Py.8) was isolated on an 8.25" X 5" preparative gel. A 0.75" strip on each side of the gel was stained with ethidium bromide to serve as a marker to isolate a strip of agarose containing the YAC. The strip was cut into 1 g blocks and washed three times for 30 minutes at 4°C in 5 ml of 20 mM Tris-HCl/1mM EDTA/500-1000 μM spermine-HCl pH8.0. The agarose block was then melted at 65°C for 15 minutes, equilibrated to 40°C, and 15 units of β-

agarase (NEB, cat. no. #392L) was then added with gentle mixing. Agarose was digested for 90 minutes at 40°C, and then either used directly to transfect ES cells, at a YAC DNA concentration of 0.1-0.5 ng/μl, or concentrated to 1-2 ng/μl by centrifugation in a Millipore Ultrafree-CL, 300,000 NMWL filter unit for 15-30 minutes at 1,000 rpm (Beckman TJ-6 centrifuge) before transfection. The integrity and purity of the YAC DNA was tested by PFGE and Southern analysis prior to transfection. The neo^r gene in this cassette is transcriptionally dependent on the mutant polyoma enhancer PyF101 and the herpes simplex virus thymidine kinase promoter, and was demonstrated to be a highly efficient vector for generating neo^r ES cell lines (unpublished observations). pHIS3PyF101neobpA was digested with ScaI within the ampicillin resistance (amp^r) gene and introduced, by lithium acetate transformation, into YPH857 carrying APP-8. Two potential arrangements following a homologous recombination event that introduces the plasmid into the amp^r gene within the TRPI arm of the YAC, are diagrammed in Figure 4A. The resulting His$^+$ transformants were tested for the integration of the neo^r plasmid by digesting with NotI and Southern analysis with probes specific for the amp^r (Figure 4B) and neo^r (Figure 4C) genes. Two APP-8 transformants, Py.2 and Py.3, (lanes 1 and 2) had the predicted fragments expected for insertion of a single copy of the integrating plasmid, whereas Py.6 and Py.8 (lanes 3 and 4) exhibited restriction patterns consistent with multiple (~5 copies) integrants in a tandem arrangement (see Figure 4A). Extensive restriction analysis revealed that all four transformants contain an unrearranged human APP gene (data not shown).

Example 3.

Introduction of APP YACs Into ES Cells

ES D3 cells (kindly provided by Dr. Tom Doetschman, University of Cincinnati) were cultured on mitomycin C-treated primary embryonic fibroblasts essentially as described (Robertson, 1987) except cells were grown in 15% fetal calf serum at 10% CO_2. Leukemia inhibitory factor (LIF) was provided by a 1:500 dilution of conditioned media from CV-1 cells that had been stably transformed with the LIF expression vector pC10-6R (Smith, et al., 1988, *Nature* (London), 336:688-690).

2-4 X 10^5 ES cells were plated in a 35-mm well 18 hours prior to transfection. 100 μl of purified YAC DNA was transferred by a wide bore capillary pipette to 2.5-15 μg of Lipofection (BRL, catalog #8292SA) diluted to 100 μl with OptiMEM (BRL, catalog # 320-1985AJ). Lipids and YAC DNA were gently mixed in polystyrene tubes, and allowed to complex for 45 minutes at room temperature. Complete ES media (0.8 ml with LIF) was added, gently mixed, transferred to media drained ES cells, and allowed to incubate for 16-18 hours. Fresh media was added, and after 36-48 hours the cells were split onto 10-cm plates in ES media supplemented with LIF and 200 μg/ml active G418. Selection was continued for 11-14 days, adding primary embryonic fibroblasts as necessary. ES line Py8.2 was picked from a plate that went through selection on a gelatinized (0.1%) plate instead of on fibroblasts. Genomic DNA was isolated from ES cells and mouse tails by a standard salting out procedure (Miller, et al., 1988, *Nucl. Acid Res.*, 161215), while high molecular weight DNA was isolated from ES cells

in agarose plugs as described (Gardiner, et al., 1986, *Somatic Cell Mol. Genetics*, 12:185-195). Human DNA was obtained from Sigma (St. Louis, MO).

Py.8, the 650 kb human APP YAC with multiple neo^r cassettes, was selected for transfection into ES cells. Although conventional strategies for introduction of YAC DNA into mammalian cells involved spheroplast fusion of the yeast carrying the YAC, the disadvantage of this method is that a majority of the yeast genome is also transferred into the recipient genome (Pachnis, et al., 1990; Pavan, et al., 1990; Huxley, et al., 1991). As an alternative, Py.8 was fractionated by PFGE and then enzymatically liberated from the agarose gel matrix with β-agarase^R. Py.8 DNA was transfected into ES cells by a lipid-mediated transfection method. Critical to this process was the inclusion of an optimal concentration of polyamines needed to stabilize the YAC DNA in solution. This procedure routinely yielded full-length purified Py.8 DNA at a concentration of 0.1-2 ng/μl. To transfer purified YAC DNA to ES cells, we also examined a number of different lipid transfection reagents including the commercially available Lipofectin^R and Transfectace^R reagents provided courtesy of Bethesda Research Laboratories. Lipofectin^R appeared to be the most effective for transferring YAC DNA to ES cells; on average, transfection of 10-200 ng of purified Py.8 YAC DNA into 1 X 10^6 ES cells with Lipofectin^r yielded 1-4 G418^r colonies. In our first transfection experiment, we generated 23 G418^r ES lines following transfection with gel-purified Py.8 DNA.

DNA PCR Analysis

The oligonucleotides used for PCR analysis are as follows: URA3 forward. 5'-AATGCACACGGTGTGGTG-3', URA3 reverse 5'

CGTCTCCCTTGTCATCTAAACC-3', promoter forward 5'-AATATCTGCTGTCCTTATAA-3', promoter reverse 5'-GACTGTTTTAGTAACCGCAT-3', exon 7 forward 5'-GTCGGTGGCCAGTTAAATTC-3', exon 7 reverse 5'-AGCAGAGTCAGTGGCGAGAG-3', exon 17 forward (as described by Goate, et al., 1991) 5'-CCTCATCCAAATGTCCCGTCATT-3', exon 17 reverse 5'-GCCTAATTCTCTCATAGTCTTAATTCCCAC-3', Xist (as described by Borsani, et al., 1991, *Nature* (London), 351:325-329) forward 5'-GGGACCTAACTGTTGGCTTTATCAG-3', and Xist reverse 5'-GAAGTGAATTGAAGTTTTGGTCTAG-3'. PCR analysis of DNA preparations were performed with 50-100 pmoles of each oligonucleotide and Taq DNA polymerase (Perkin Elmer/Cetus, Emeryville, California) for 30 cycles at an annealing temperature of 60°C. Products were fractionated on 2% agarose gels and visualized with ethidium bromide. PCR products are 277 bp for the URA3 primers, 400 bp for the human APP promoter oligos, 411 bp for the intronic human exon 7 primers, 319 bp for the intronic human exon 17 primers, and 203 bp for the Xist primers.

PCR analysis revealed that three of the 23 G418^r lines appeared to have integrated the human APP YAC, two of which are shown in Figure 5; ES lines Py8.2 (lane 6) and Py8.9 (lane 8) contained the APP human promoter (5B), exon 7 (5C), and exon 17 (5D). Py8.9 also contained the URA3 marker from the arm of the YAC farthest from the neo^r cassette (5A). Genomic DNA isolated from Py8.2 and Py8.9, as well as the other DNA positive line Py8.29, was subjected to extensive restriction analysis, revealing that the entire human APP gene was

essentially intact and unrearranged (data not shown). Furthermore, high molecular weight DNA was isolated from the ES lines containing the APP YAC, digested with HindIII and probed with a human Alu repetitive element (Figure 6). This analysis reveals a characteristic Alu profile of the 650 kb Py.8 YAC (lane 1), and an essentially indistinguishable pattern in ES lines Py8.2 (lane 5) and Py8.9 (lane 6), strongly suggesting that in these lines the YAC was transferred intact. On the other hand, Py8.29, appeared to be lacking several Alu elements (lane 7, arrows). A comparison of hybridization intensities between endogenous mouse APP and human APP suggested that lines Py8.2 and Py8.29 contain a single copy of the APP YAC, whereas line Py8.9 contains multiple copies (data not shown). The transfection protocol described here is highly reliable and reproducible, as an independent transfection of ES cells with Py.8 YAC DNA yielded 11 G418^r lines, one of which appeared to contain the entire human APP YAC, and transfection of ES cells with a YAC containing the human superoxide dismutase I (SODI) gene from chromosome 21, has generated several ES lines containing the YAC (data not shown).

Fluorescent In-Situ Hybridization

Mouse embryonic stem cell metaphase chromosomes were prepared using a mitotic shake off procedure according to standard cytogenetic protocols. 100 ng of human COT-1 DNA was labelled with Bioltin-14-dATP by nick translation (Langer, et al., 1981, *Proc. Natl. Acad. Sci. USA*, 78:6633-6637) and hybridized to mouse chromosome and interphase nuclei preparations. Hybridization and signal detection was accomplished as described previously (Kievits, et al., 1990, *Cytogenet. Cell Genet.*, 53:134-136). Chromosomes were counterstained with

propidium iodide (0.5 μg/ml) and DAPI (0.5 μg/ml) in antifade medium (Johnson and Araujo, 1981, *J. Immuno. Methods*, 43:349-350) and viewed using a Leitz Aristoplan epi-fluorescent microscope equipped with a double band pass filter (FITC/Texas Red).

Finally, we used fluorescent in-situ hybridization (FISH) of the chromosomes from ES lines Py8.2 and Py8.9 to demonstrate integration of the YAC. FISH with human repetitive COT-1 DNA revealed a distinct signal on a single mouse chromosome in 70 out of 89 (79% efficiency) metaphase chromosome spreads from ES line Py8.2 (Figure 7A) and 114 out of 130 (88% efficiency) interphase nuclei contained a single signal, strongly suggesting the YAC integrated into a single mouse chromosome in ES line Py8.2. On the other hand, ES line Py8.9 demonstrated a distinct signal on two mouse chromosomes in 62 out of 75 (83% efficiency) metaphase chromosome spreads (Figure 7B), and 111 out of 125 (89% efficiency) interphase nuclei contained two signals, suggesting the YAC had integrated into two different mouse chromosome in ES line Py8.9. As controls, untransfected ES cell metaphase chromosome spreads exhibited no signal with human COT-1 DNA, while human metaphase chromosomes exhibited signal on every chromosome (data not shown).

RNA Analysis

For Northern blot analysis of ES cells, cytoplasmic RNA was prepared as described (Sisodia, et al., 1987, *Mol. Cell. Biol.*, 7:3602-3612 RNA was fractionated by electrophoresis on formaldehyde-agarose gels and then transferred to nitrocellulose membranes. Membranes were hybridized with a full-length human APP-695 cDNA in 50% formamide/6XSSC at 42°C and then washed at

0.1XSSC at 50°C. Blots were stripped, and then reprobed with Mβ5, a cDNA encoding mouse class IV β-tubulin. Probes were labeled with [^{32}P] using a random primer-mediated protocol (Feinberg and Vogelstein, 1984, *Analytical Biochemistry*, Addendum, 137:266-267) to specific activities of between 1.5-2.5 X 10^9 cpm/μg.

Example 4.

Expression of human APP in ES cells

The ES lines containing the integrated human APP YAC were examined for human APP mRNA expression and synthesis of human APP. We prepared cytoplasmic RNA from untransfected ED D3 cells (Figure 8, lane 1), ES cells lines containing the APP YAC (Figure 8, lanes 2-4), and D3 cells transfected with pHIS3PyF101neobpA (Figure 8, lane 5). RNA was fractionated by denaturing gel electrophoresis, transferred to a nitrocellulose membrane, and then probed with a full-length APP-695 cDNA (Figure 8), a probe which recognizes human and mouse mRNA with equal efficiency (Sisodia and Lamb, unpublished observations). Our studies document that ES lines Py8.2, Py8.9, and py8.29 accumulate full length APP mRNA, with the highest expression in line Py8.2 (lane 2), moderate levels in line Py8.9 (lane 3), and low but detectable levels in line Py8.29 (lane 4), as compared to untransfected ES cells (lane 1), and to ES cells transfected with pHIS3PyF101neobpA (lane 5). Notably, mouse APP mRNA is expressed at extremely low levels in ES cells (lanes 1 and 5). These findings were confirmed by S1 nuclease protection assays and reverse transcriptase PCR (RT-PCR), which indicated that human APP mRNA in all three ES lines was correctly initiated and spliced (data not shown). It is presently uncertain whether the observed

differences in the levels of expression of the three lines can be attributed to disparate chromosomal integration sites or other factors.

Protein Analysis

Protein extracts from cells and tissue were prepared for immunoblot by lysis and homogenization in 1X Laemmli buffer (2% SDS/62.5 mM Tris-HCl, pH 6.8/5% β-mercaptoethanol/20% glycerol), boiling, and then fractionated by SDS-PAGE on 6% polacryamide gels, prior to electrophoretic transfer to nitrocellulose.

To examine the expression of human APP in the Es lines we performed Western blot analysis with a polyclonal antibody, B5, generated against human APP-695 residues 444 to 591 (Athena Neuroscience, South San Francisco, CA). In Figure 9, we document accumulation of human APP in ES line Py8.2 (lane 4), with little, if any expression in lines Py8.9 and Py8.29 (lanes 5 and 6). These results were confirmed by labelling cells with [^{35}S] methionine and then immunoprecipitating with the same antibody (data not shown). These results indicate that a large APP YAC stably integrated into ES cell DNA generates accurately initiated and correctly spliced human APP transcripts and the concomitant expression of human APP.

Example 5.

Transmission of APP YACs into the Mouse Germline

Cells of ES lines Py8.2 and Py8.9 were injected into blastocysts derived from C57B1/6J mice in order to generate chimeric mice. Five of the Py8.9 chimeras (all male) and five of the Py8.2 chimeras (one female and four males) have produced offspring with agouti coat color, indicating the successful transmission of ES cell DNA through the germline.

All mice were obtained from the Jackson Laboratory. Chimeric mice were generated by injection of embryonic stem cells into C57BL/6J blastocysts and transfer of the injected embryos into the uterus of pseudopregnant female CD-1/VAF mice (Bradley, 1987). ES cells (n=12-15) were injected into each embryo and 10-20 injected embryos were transferred to each pseudopregnant female. For ES line Py8.9, a total of 107 embryos were transferred to several pseudopregnant females and 26 pups were born (24.3%), including nine male chimeras (34.6%). These chimeras were mated to C57Bl/6J mice, and transmission of the ES cell component judged by the presence of agouti coat color. Five Py8.9 chimeras (all male) have produced agouti offspring. For ES line Py8.2, a total of 110 embryos were transferred to six pseudopregnant females and 69 pups were born (62.7%), including seven male and five female chimeras (15.9%). Five (one female and four males) Py8.2, chimeras have produced agouti offspring.

A number of the agouti offspring from Py8.9 chimeras were examined for the presence of YAC DNA and for expression of human APP. PCR analysis of tail DNA from the first eight agouti pups revealed that three animals (Figure 10, lanes 12, 14, and 15) contained the human APP promoter (Figure 10B), exon 7 (Figure 10C), and exon 17 (Figure 10D). Only one of these three animals, however, contained the URA3 marker from the arm of the YAC (Figure 10A). These results suggest that there are two different populations of DNA in these mice, consistent with the FISH (Figure 7B) demonstrating two different integration sites in line Py8.9. DNA positive agouti offspring from Py8.2 chimeras have also been identified (data not shown). Mice containing the human APP YAC appear

in brains among three independent transgenic animals derived from Py8.9 chimeras are indistinguishable (Figure 11C).

Reverse Transcription PCR (RT-PCR) Analysis

RNA was isolated from mouse brain, heart, kidney, and testes by homogenization of tissue in quinidine thiocyanate and centrifugation through a CsCl cushion. Each RNA preparation (1 μg) was reverse transcribed in the presence of 50 pmol random hexamer primers (pd$^{(\alpha)}$6; Pharmacia, Piscataway, New Jersey) and Reverse Transcriptase™ (Bethesda Research Laboratories, Bethesda, Maryland). For analysis of relative levels of total human APP transcripts to mouse APP transcripts in each RNA preparation, 1/25 of the reverse transcription reaction was incubated in a PCR with 50 pmol of each primer, Hu/Mo APP (S) and Hu/Mo APP (AS), for 20 cycles at an annealing temperature of 60°C.

The sequence of Hu/Mo APP (S) and Hu/Mo APP (AS) are 5'-GTCRCGATGCTGCCRGYTTGG-3', and 5'-TSGGCARCAYACAAACYCYACC-3' respectively, where R=A+G, Y=C+T, and S=G+C. Hu/Mo APP (S) encodes amino acids 1-6 of APP. The RT-PCR product of 568 bp spans the first six exons of APP.

Included in the reaction was pmol Hu/Mo APP (S), 5' end labeled with γ[^{32}P] ATP and T4 polynucleotide kinase. PCR products were purified and then digested with SphI, which digests the mouse APP product uniquely. Resulting products were fractioned on 2% agarose gels, stained with ethidium bromide, photographed, and then dried and exposed to Kodak X-Omat film.

For analysis of the relative levels of human and mouse APP transcripts encoding KPI+ and KPI- APP isoforms, 1/25 of the reverse transcription reaction was incubated in a PCR with 50 pmol of each primer, Hu/Mo KPI (S) and Hu/Mo KPI (AS), for 20 cycles at an annealing temperature of 60°C. The sequence of Hu/Mo KPI (S) and Hu/Mo KPI (AS) is 5'-GACARYGTGGATTCTGCKGATGC-3' and 5'-GGATAACKGCCTTCTTRTCAGC-3' respectively, where R=A+G, Y=C+T, and K=G+T. Hu/Mo KPI (AS) is complementary to sequences that encode amino acids 351 to 358 of APP. Included in the reaction mixture was 1 pmol of [^{32}P] end-labeled Hu/Mo KPI (S). PCR products were purified and then digested with SphI, which digests the mouse APP products uniquely. Resulting products were fractionated on 2% agarose gels, stained with ethidium bromide, photographed, and then dried and exposed to Kodak X-Omat film.

The expression of alternatively spliced APP mRNA in the APP YAC transgenic mouse C9.24 and the age-matched control mouse C9.20 was also examined by quantitative RT-PCR. RT-PCR was performed with denerate primers corresponding to highly conserved, identically positioned sequences in human and mouse APP cDNA that flank the KPI-encoding exons and included trace amounts of [^{32}P] labeled sense primer. Thus, PCR amplification gives rise to labeled products 490, 658, and 715 bp representing APP-695, 751, and -770 transcripts respectively. Digestion of the PCR products with SphI specifically cleaves mouse APP products and generates labeled products of 360, 528, and 585 bp (Figure 12A), leaving the human products uncut. Figure 12B depicts fragments resulting from SphI digestion of RT-PCR products generated from transgenic mouse C9.24 and control mouse C9.20 tissues. It is apparent that the relative levels of products

representing alternatively spliced human APP transcripts in the transgenic animal parallels that observed for the mouse APP transcripts in the age-matched control. For example, the relative ratio of mouse APP-695, -751, and -770 transcripts in C9.20 brain (Figure 12B, lane 1, arrowhead) is essentially comparable to the ratio of human APP-695, -751, -770 transcripts in C9.24 brain (Figure 12B, lane 5, asterisk). Additionally, we have recently obtained an APP YAC transgenic mouse from a Py8.2 chimera that coexpresses human and mouse APP mRNA. In summary, APP YAC transgenic mice express the different human APP transcripts in levels and in tissues comparable to the endogenous mouse APP transcripts.

The expression of human APP in tissues of APP YAC transgenic mice derived from Py8.9 chimeras was examined by Western blotting utilizing antibody B5, raised against human APP (Figure 13A). Substantial levels of human APP expression was observed in the brain, heart, kidney, and testes of APP YAC transgenic mouse C9.24 (lanes 6-9), as compared to the age-matched control, C9.20 (lanes 2-5). Notably, B5 appears to be highly specific for human APP, as virtually no signal was obtained in tissues of the control animal. To assess whether levels of total APP in transgenic tissue was elevated over control mouse tissues, we reprobed the above samples with the polyclonal antibody CT15, raised against the common terminal 15 residues of mouse/human APP (Figure 13B). Although APP levels appear to be somewhat elevated in brain extracts of the transgenic mouse, this pattern may reflect disparate loading, because reprobing the blot with the β-tubulin-specific monoclonal antibody DM13 showed slightly higher β-tubulin immunoreactivity in the C9.24 brain as compared to the C9.20 control brain (data not shown). Preliminary immunocytochemical studies with the human

APP-specific antibody B5 in a six-week transgenic mouse brain have documented strong staining in neurons, consistent with other *in situ* and immunocytochemical studies (data not shown). However, as expected, we have not detected any evidence for pathological or cellular alterations in these young animals.

CLAIMS

1. A method for producing a transgenic animal containing a contiguous foreign DNA segment of at least about 70,000 base pairs (70 kb), comprising:

(a) integrating a selectable resistance cassette into a yeast artificial chromosome containing said contiguous foreign DNA segment, said selectable resistance cassette containing a first selectable marker which is expressed in yeast and a second selectable marker which is expressed in mammalian cells;

(b) purifying said yeast artificial chromosome containing said contiguous foreign DNA segment and said selectable resistance cassette from other yeast DNA sequences;

(c) introducing said yeast artificial chromosome containing said contiguous foreign DNA segment and said selectable resistance cassette into embryonic stem cells;

(d) selecting a clone of embryonic stem cells containing said contiguous foreign DNA segment;

(e) injecting embryonic stem cells of said clone into blastocysts of the same species; and

(f) generating transgenic animals from said injected blastocysts.

2. The method of claim 1 wherein the transgenic animal is a mouse.

3. The method of claim 1, wherein the contiguous foreign DNA segment is a contiguous segment of foreign genomic DNA of at least about 100 kb.

4. A method of producing an embryonic stem cell containing a contiguous foreign DNA segment of at least about 70 kb, comprising

(a) integrating a selectable resistance cassette into a yeast artificial chromosome containing said contiguous foreign DNA segment, said selectable resistance cassette containing a first selectable marker which is expressed in yeast and a second selectable marker which is expressed in mammalian cells;

(b) purifying said yeast artificial chromosome containing said contiguous foreign DNA segment and said selectable resistance cassette from other yeast DNA sequences;

(c) introducing said yeast artificial chromosome containing said contiguous foreign DNA segment and said selectable resistance cassette into embryonic stem cells;

(d) selecting a clone of embryonic stem cells containing said contiguous foreign DNA segment.

5. The method of claim 4, wherein the contiguous foreign DNA segment is a contiguous segment of foreign genomic DNA of at least about 100 kb.

6. A transgenic mouse containing at least about 400 kb of human genomic DNA, said DNA comprising the amyloid precursor protein (APP) promoter and 18 exons and the intervening sequences of the APP sequence.

7. The transgenic mouse of claim 6, wherein human APP is expressed.

8. The transgenic mouse of claim 7, wherein the APP sequence encodes APP-770 having in position 717 an amino acid residue selected from the group consisting of isoleucine, phenylalanine and glycine.

9. The transgenic mouse of claim 7, wherein the APP sequence encodes APP-770 having in positions 670 and 671 the amino acid residues asparagine and leucine, respectively.

10. The transgenic mouse of claim 6, containing a DNA sequence corresponding to the sequence of a yeast artificial chromosome selected from the group consisting of APP-2, APP-5, APP-7 and APP-8.

11. The transgenic mouse of claim 7 wherein the APP sequence encodes APP-770 having glycine in position 692.

12 The transgenic mouse of claim 7 wherein the APP sequence encodes APP-770 having glutamine in position 693.

Figure 1

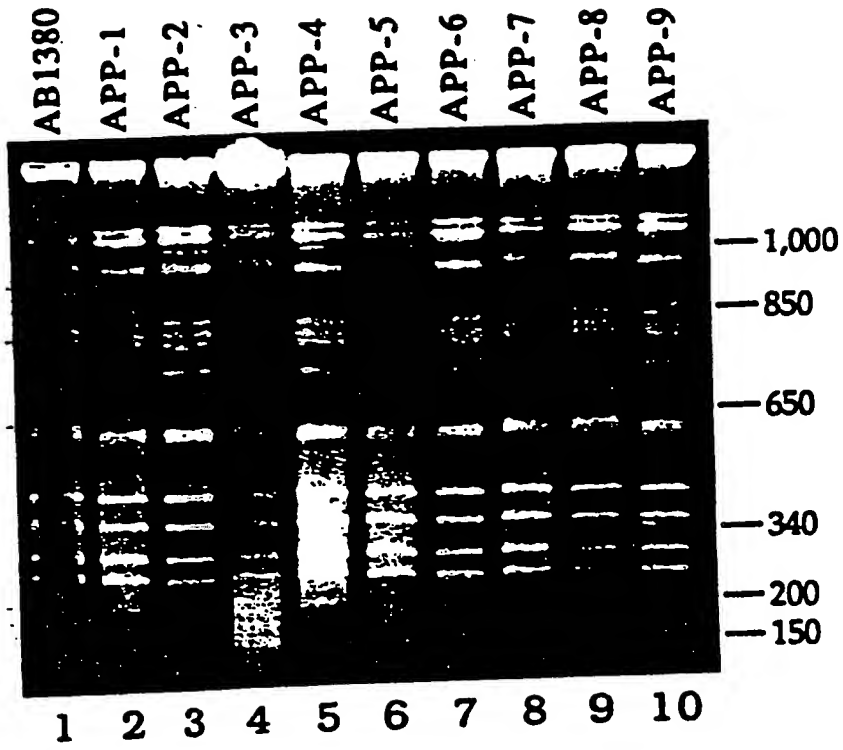

AB1380
APP-1
APP-2
APP-3
APP-4
APP-5
APP-6
APP-7
APP-8
APP-9
1,000
850
650
340
200
150
1 2 3 4 5 6 7 8 9 10

Figure 2

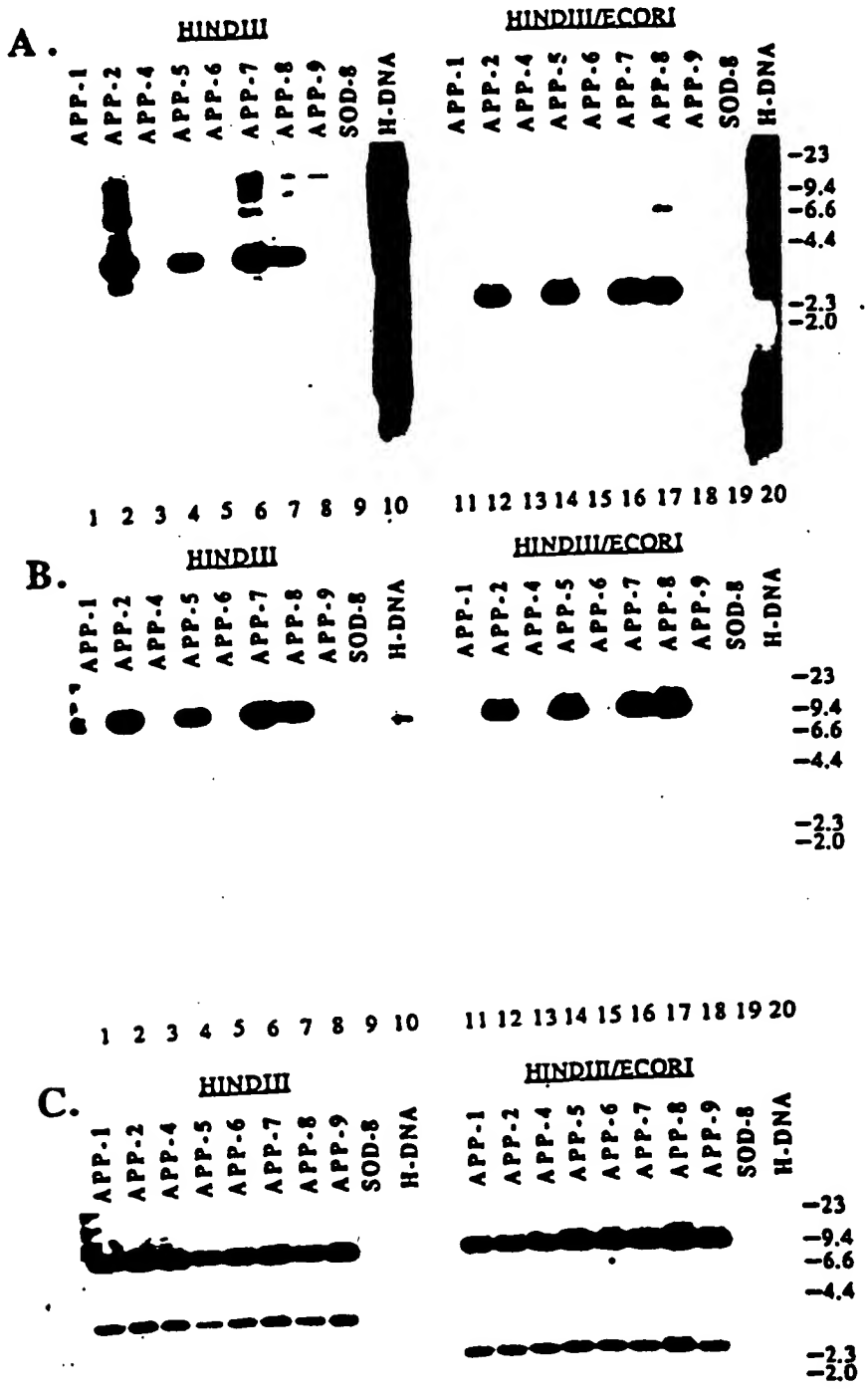
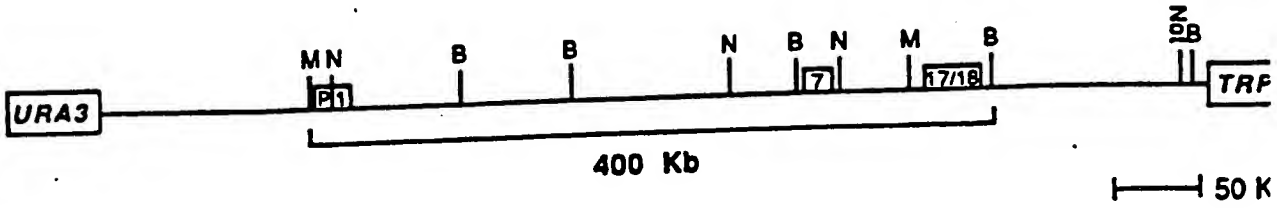

A.
HINDIII
HINDIII/ECORI
APP-1
APP-2
APP-4
APP-5
APP-6
APP-7
APP-8
APP-9
SOD-8
H-DNA
-23
-9.4
-6.6
-4.4
-2.3
-2.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
B.
HINDIII
HINDIII/ECORI
C.
HINDIII
HINDIII/ECORI

Figure3

A.


MN
B
B
N
B N
M
B
NotI
B
URA3
P1
7
17/18
TRF
400 Kb
50 K

B.

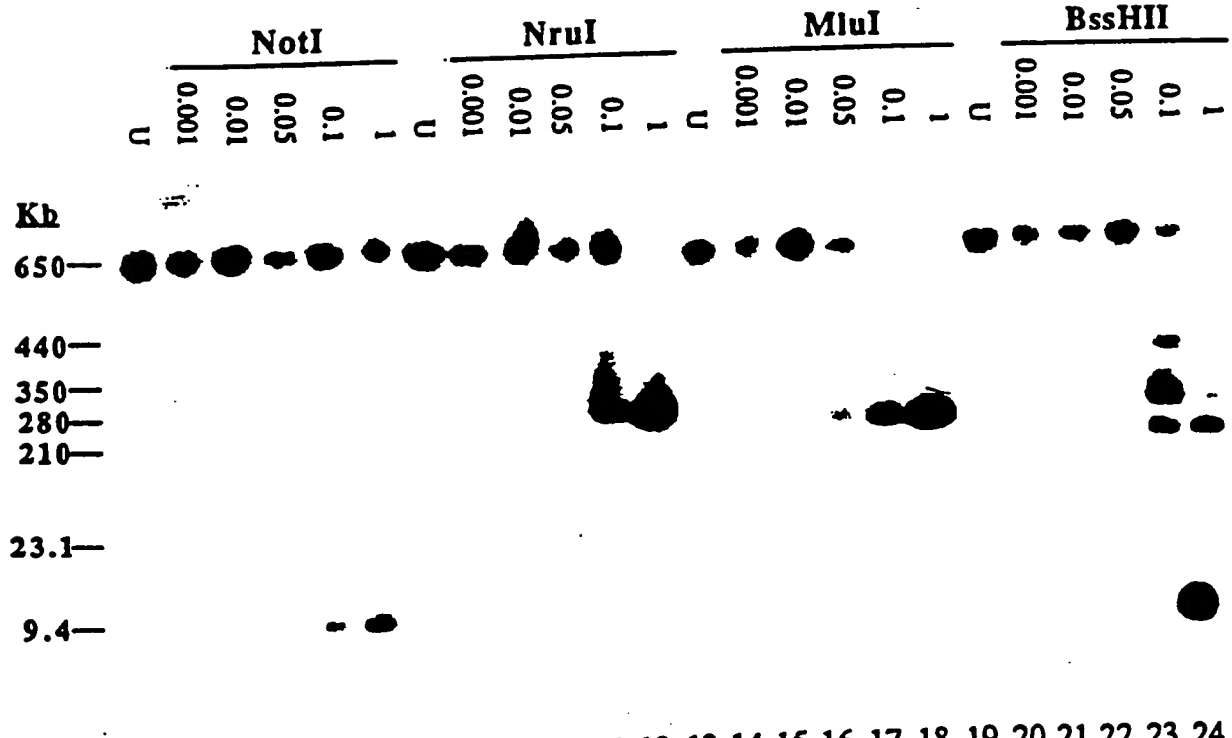
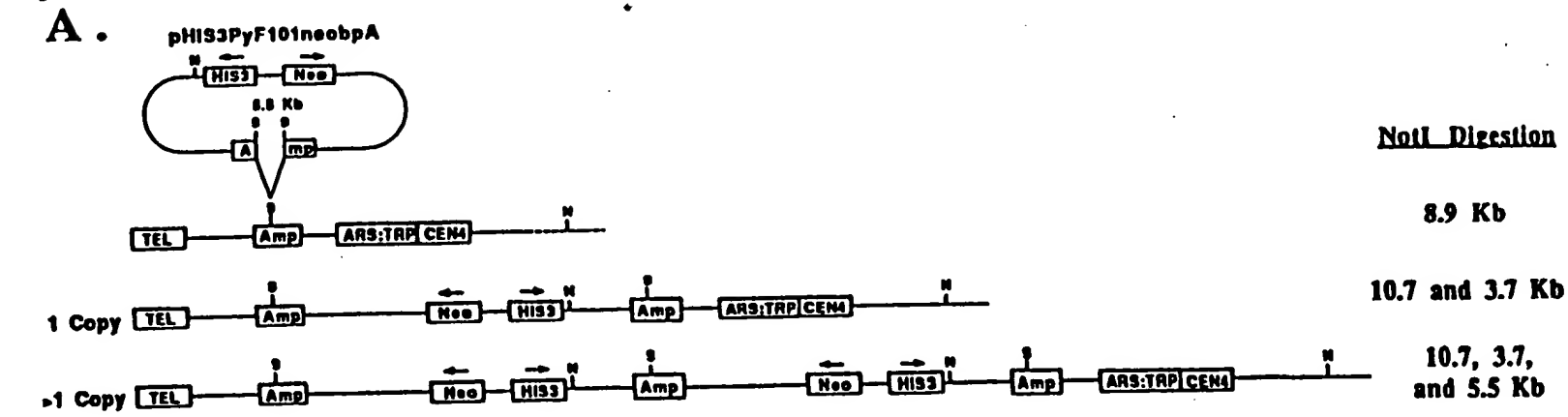
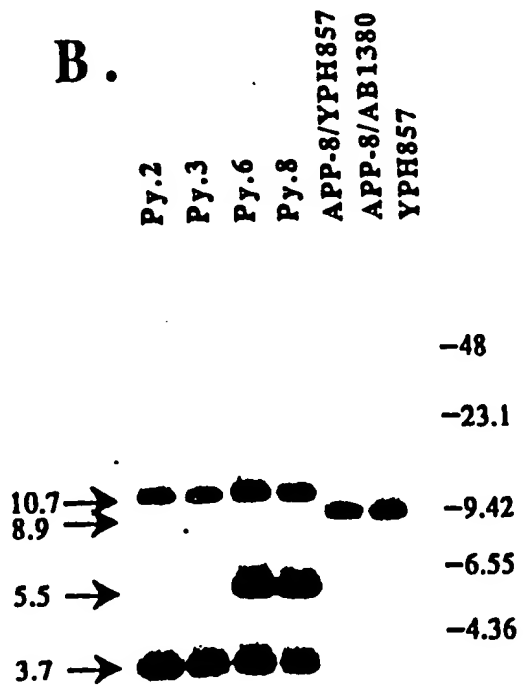
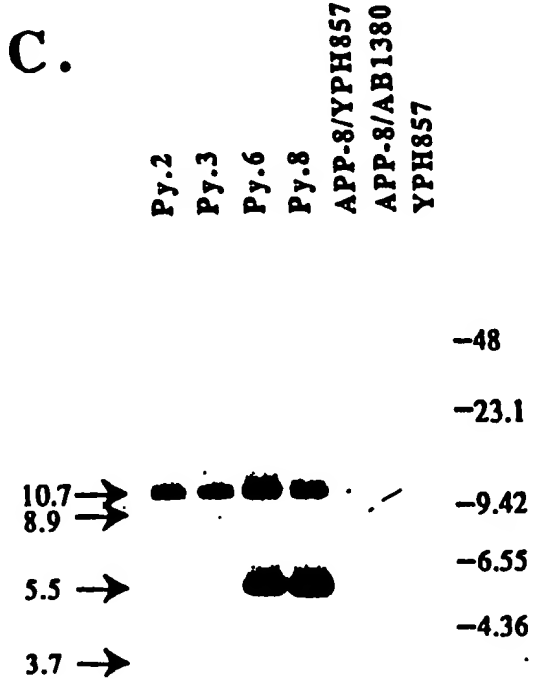

NotI
NruI
MluI
BssHII
U
0.001
0.01
0.05
0.1
1
Kb
650
440
350
280
210
23.1
9.4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Figure 4


A.
pHIS3PyF101neobpA
HIS3
Neo
8.8 Kb
A
mp
TEL
Amp
ARS:TRP
CEN4
1 Copy
>1 Copy
NotI Digestion
8.9 Kb
10.7 and 3.7 Kb
10.7, 3.7, and 5.5 Kb


B.
Py.2
Py.3
Py.6
Py.8
APP-8/YPH857
APP-8/AB1380
YPH857
10.7
8.9
5.5
3.7
48
23.1
9.42
6.55
4.36


C.
Py.2
Py.3
Py.6
Py.8
APP-8/YPH857
APP-8/AB1380
YPH857
10.7
8.9
5.5
3.7
48
23.1
9.42
6.55
4.36

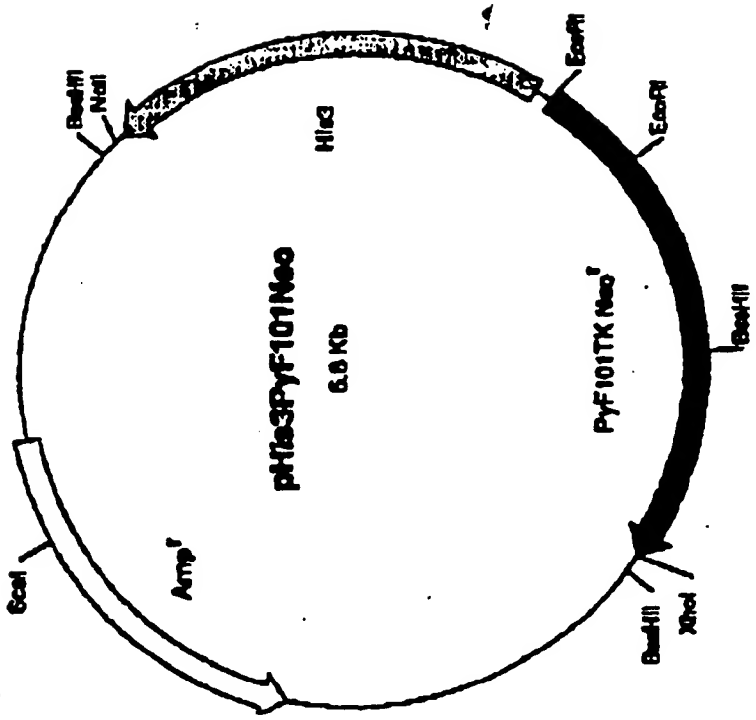

BamHI
NdeI
HindIII
EcoRI
EcoRI
pHis3PyF101Neo
6.8 Kb
PyF101TK Neo
BamHI
EcoRI
Amp
BamHI
XhoI

FIGURE 4D

Figure 5

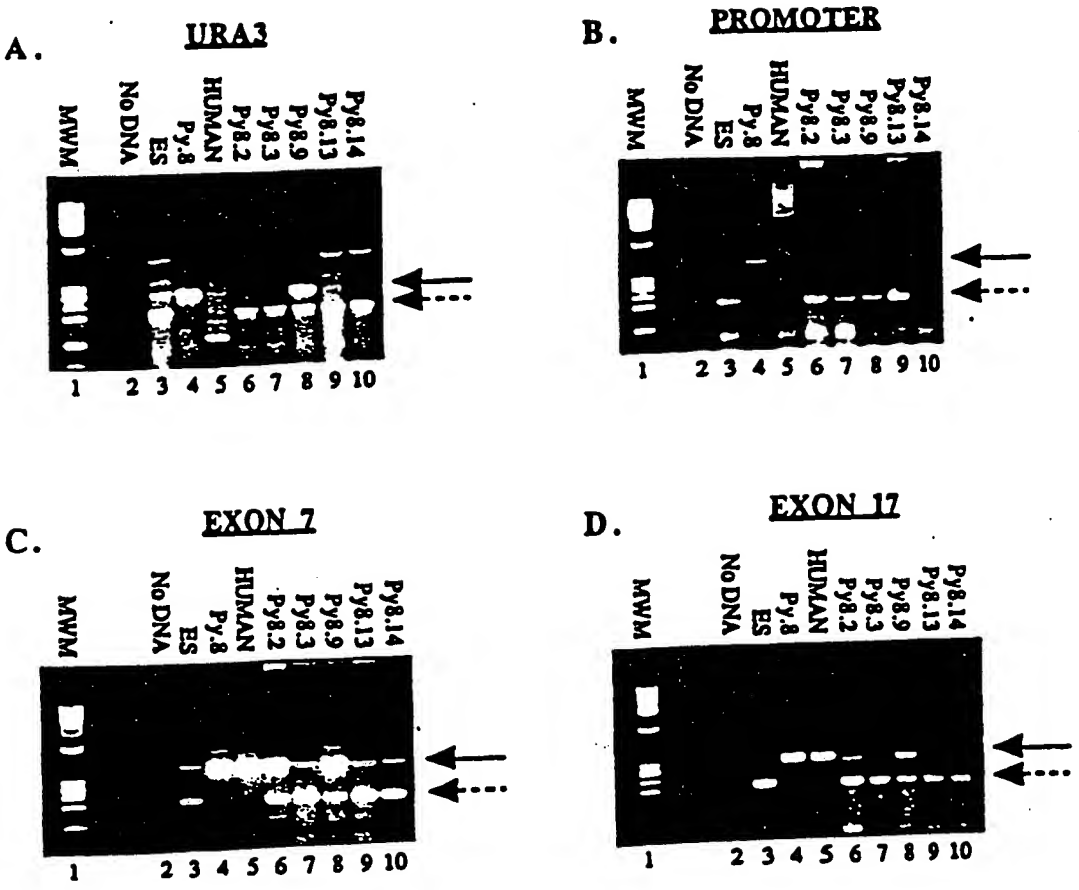

A.
URA3
MWM
No DNA
ES
Py.8
HUMAN
Py8.2
Py8.3
Py8.9
Py8.13
Py8.14
1 2 3 4 5 6 7 8 9 10
B.
PROMOTER
MWM
No DNA
ES
Py.8
HUMAN
Py8.2
Py8.3
Py8.9
Py8.13
Py8.14
1 2 3 4 5 6 7 8 9 10
C.
EXON 7
MWM
No DNA
ES
Py.8
HUMAN
Py8.2
Py8.3
Py8.9
Py8.13
Py8.14
1 2 3 4 5 6 7 8 9 10
D.
EXON 17
MWM
No DNA
ES
Py.8
HUMAN
Py8.2
Py8.3
Py8.9
Py8.13
Py8.14
1 2 3 4 5 6 7 8 9 10

Figure 6

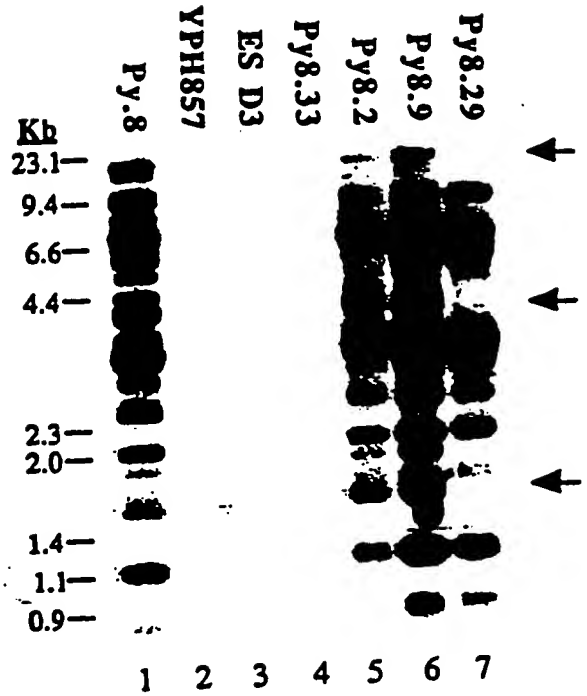
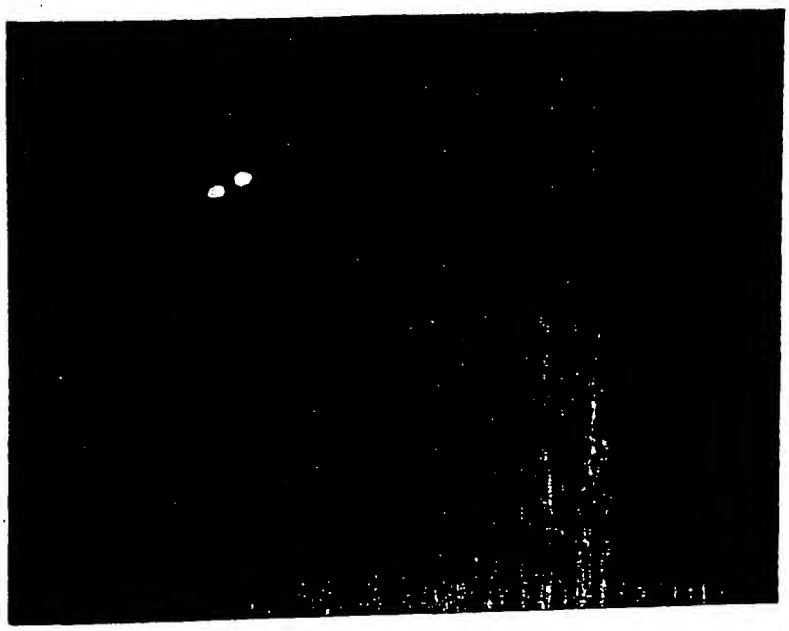

Kb
23.1
9.4
6.6
4.4
2.3
2.0
1.4
1.1
0.9
Py.8
YPH857
ES D3
Py8.33
Py8.2
Py8.9
Py8.29
1 2 3 4 5 6 7

Figure 7

A.



B.

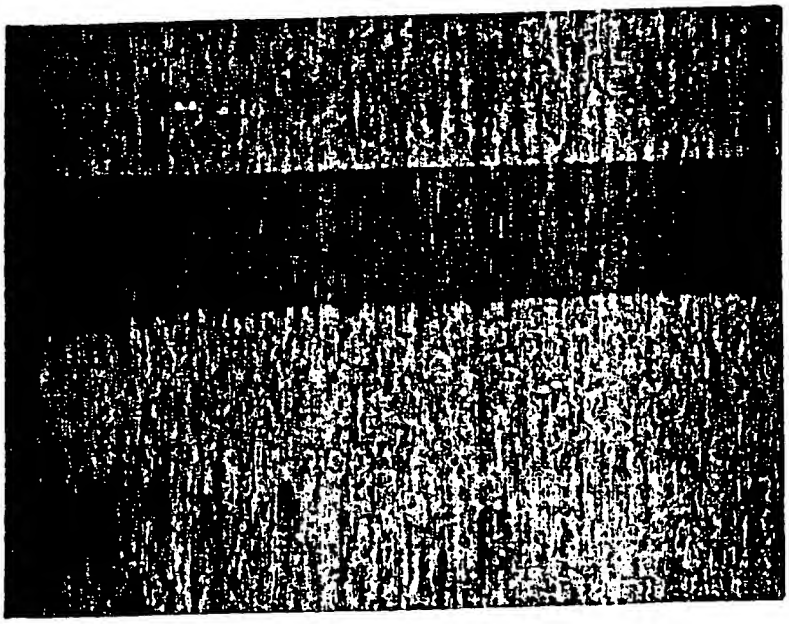


Figure 8

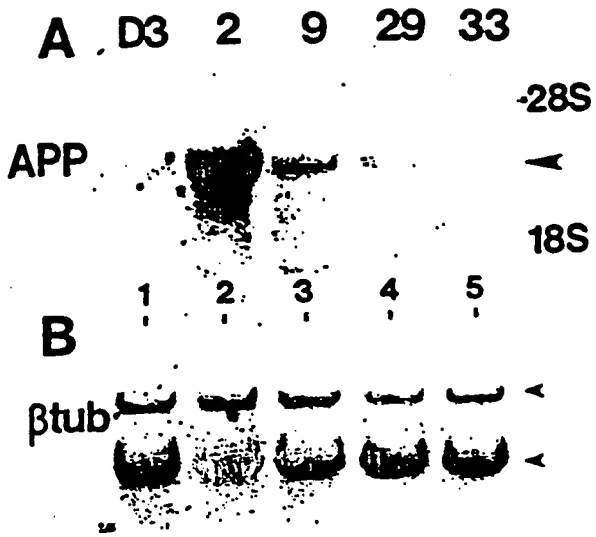


Figure 9

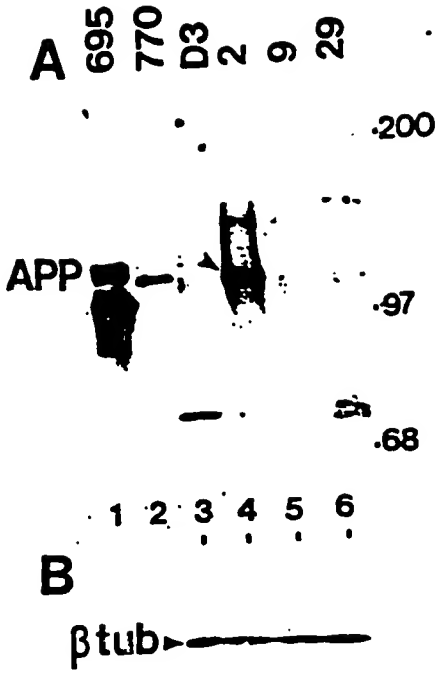
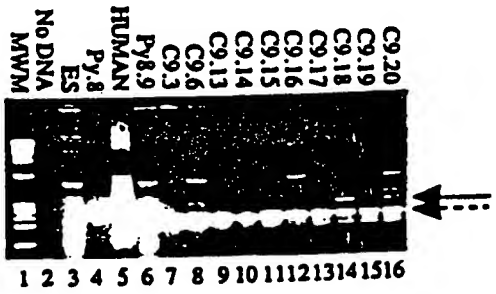
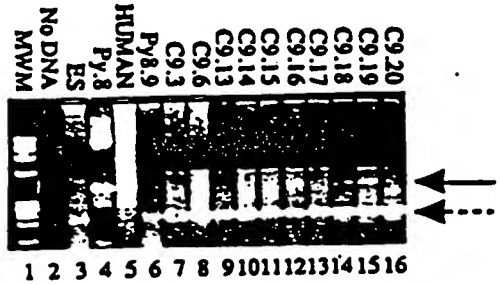


Figure 10

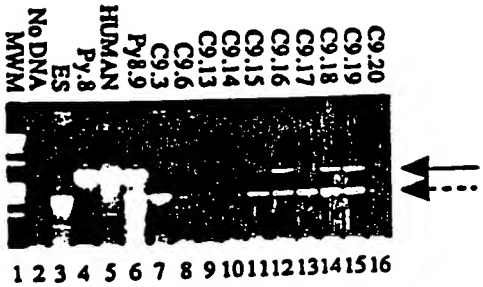
A. URA3



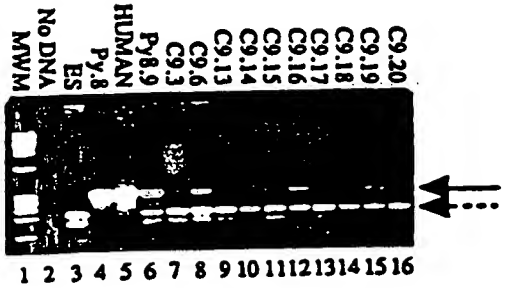
B. PROMOTER



C. EXON 7



D. EXON 17



Bigure 11

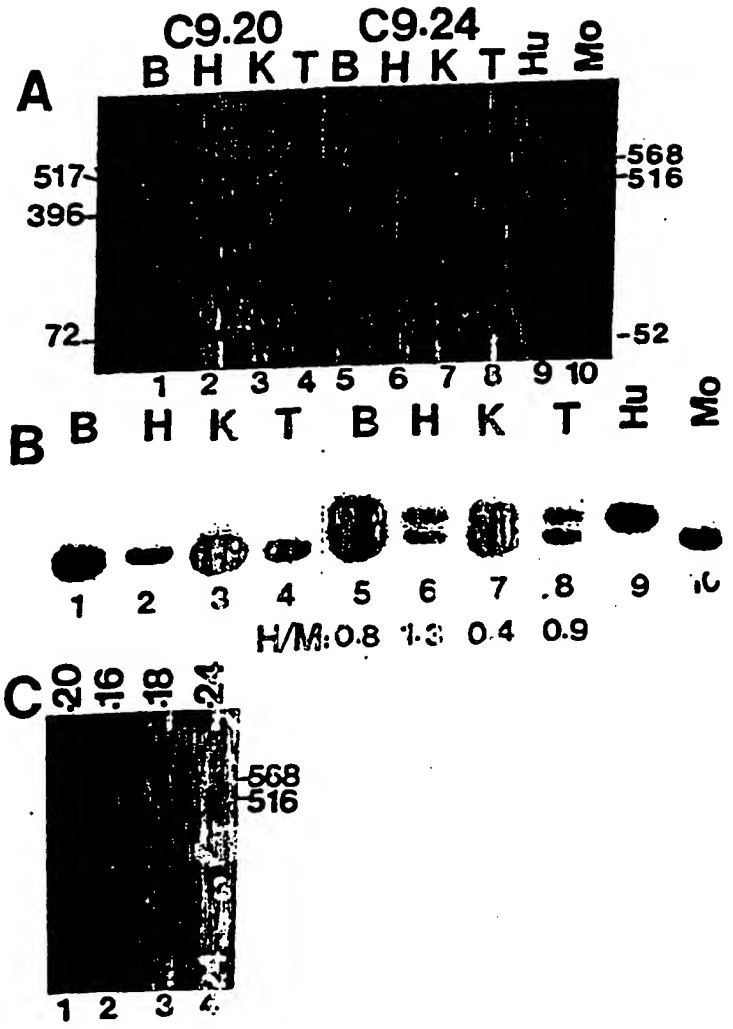

A
C9.20
C9.24
B H K T B H K T Hu Mo
517
396
72
568
516
52
1 2 3 4 5 6 7 8 9 10
B
B H K T B H K T Hu Mo
1 2 3 4 5 6 7 8 9
H/M: 0.8 1.3 0.4 0.9
C
20 16 18 24
568
516
1 2 3 4

Figure 12

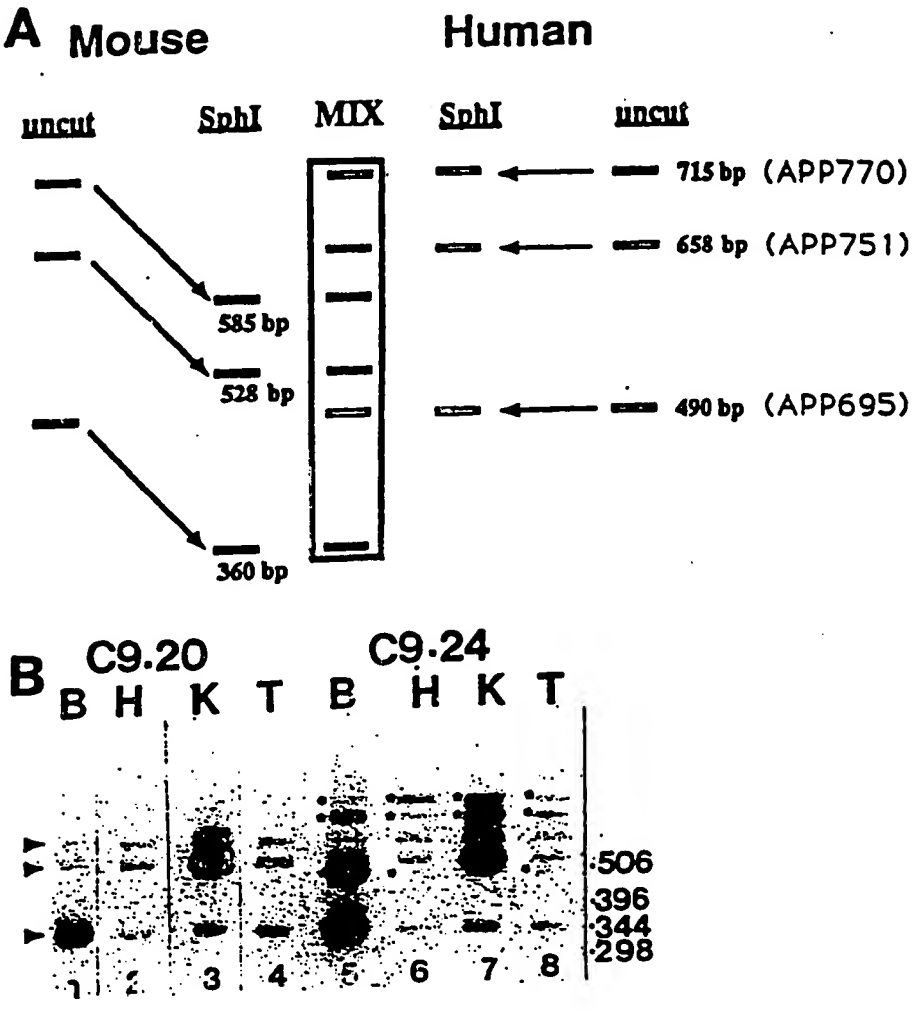

A
Mouse
Human
uncut
SphI
MIX
SphI
uncut
715 bp (APP770)
658 bp (APP751)
585 bp
528 bp
490 bp (APP695)
360 bp
B
C9.20
C9.24
B H K T B H K T
506
396
344
298
1 2 3 4 5 6 7 8

Figure 13